



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/56, 9/24, A23K 1/165		A1	(11) International Publication Number: WO 00/29587
			(43) International Publication Date: 25 May 2000 (25.05.00)
(21) International Application Number: PCT/CA99/01093		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 16 November 1999 (16.11.99)			
(30) Priority Data: 60/108,504 16 November 1998 (16.11.98) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US Filed on Not furnished (CON) Not furnished			
(71) Applicant (for all designated States except US): IOGEN CORPORATION [CA/CA]; 400 Hunt Club Road, Ottawa, Ontario K1V 1C1 (CA).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only): SUNG, Wing, L. [CA/CA]; 2148 Fillmore Crescent, Gloucester, Ontario K1J 6A4 (CA). TOLAN, Jeffrey, S. [CA/CA]; 114 Hawk Crescent, Ottawa, Ontario K1V 9G7 (CA).			
(74) Agents: SECHLEY, Konrad, A. et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).			

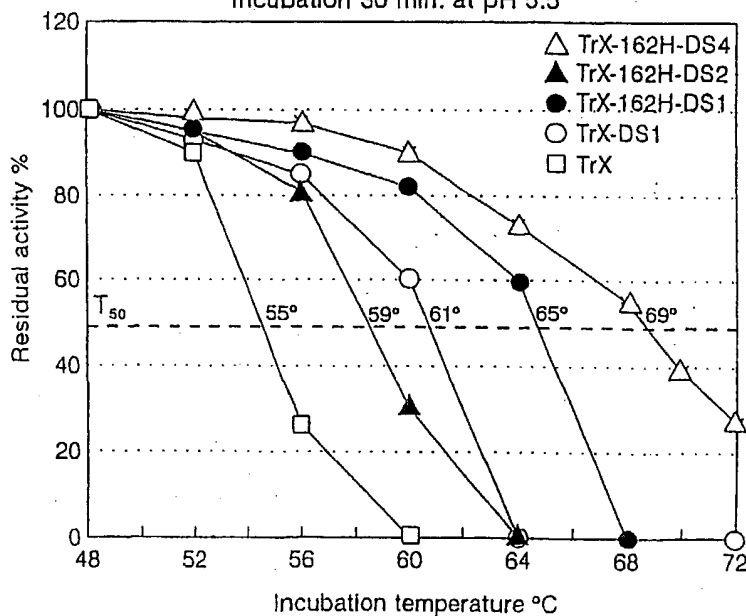
(54) Title: THERMOSTABLE XYLANASES

(57) Abstract

The present invention is directed to thermostable xylanase enzymes that are suitable for feed pelleting applications. The novel xylanase enzymes comprise at least 40 % of their optimal activity from a pH range from about pH 3.5 to about pH 6.0, and from about 40 to about 60 °C, and exhibit at least 30 % of their optimal activity after a pre-incubation step for 30 minutes at 70 °C in the presence of 40 % glycerol. Also disclosed are modified xylanase molecules comprising either a basic amino acid at position 162 (TrX numbering), or its equivalent position in other xylanase molecules, at least one disulfide bridge, or a combination thereof. The thermostable xylanase molecules of the present invention have a physiological temperature and pH optima and are useful as animal feeds additives since they can withstand the heat associated with feed sterilization and pellet formation, yet they exhibit optimal activity within an animal to aid in breakdown of ingested feed.

Thermostability of mutant TrX

Incubation 30 min. at pH 5.5



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

THERMOSTABLE XYLANASES

5 The present invention relates to thermostable xylanase enzymes. More specifically, the present invention is directed to thermostable xylanase enzymes that exhibit high activity at or near physiological pH and temperature, and their use in feed pelleting applications.

BACKGROUND OF THE INVENTION

10

Natural xylanase enzymes, such as that of the fungus *Trichoderma reesei*, have been added to animal feed to increase the efficiency of digestion and assimilation of nutrients. During digestion of feed grains such as wheat and barley, non-starch polysaccharides, including xylan, increases the viscosity of the digesta in the absence of added exogenous enzyme. This interferes with the diffusion of the digestive enzymes to the feed and the subsequent assimilation of the nutrients. The highly viscous digesta increases the occurrence of sticky stool, which increases the likelihood of disease and causes effluent run-off problems. The addition of xylanase in feed breaks down the xylan and decreases the viscosity of the digesta, thereby helping to alleviate these problems. Xylanase produces a cost saving by increasing the efficiency of feed conversion. Xylanase can decrease the feed consumed/ weight gain ratio by 5-15% (Viveros, A., Brenes, A., Pizarro, M. and Castano, M., 1994, Animal Feed Sci. Technol. 48:237-251).

20

25 Xylanase enzymes used for feed are typically aqueous solutions of active protein, stabilizers, preservatives and other additives. The enzymes are typically sprayed onto the feed at concentration of 100-2000 ml per tonne feed. Alternatively, granular or powdered xylanase can be used. Once the feed is consumed by the animal, the enzyme acts on xylan as the feed is ingested and digested in the gut. Eventually the xylanase, a protein molecule, is hydrolysed by the digestive enzymes (proteases) into amino acids like any protein in the feed.

30

SUBSTITUTE SHEET (RULE 26)

Increasingly, animal feeds are pelleted at high temperatures for sterilization against harmful bacteria, for example *Salmonella*. Feed pelleting is carried out by heating the feed solids with 100 to 140°C steam and passing them through an extruder/pelleting auger to form feed pellets, which then cool in a storage bin. The
5 typical time required for the material to pass through the system is 30 minutes. As is known in the art, higher temperatures can be used with shorter pelleting times, and lower temperatures with longer pelleting times, provided that the necessary moisture levels are obtained. The overall resulting temperature within the solids, prior to, during, and after pellet formation reaches about 70-95°C, for up to 30 min. It is
10 desirable to add the xylanase during the feed pelleting process. This would save the feed formulators the additional step of adding liquid xylanase, which is inconvenient and can introduce microbial contamination into the feed. The option of adding solid xylanase as a separate step is also undesirable, as the solids would not be evenly mixed. Marquardt and Bedford (1997, *Enzymes in Poultry and Swine Nutrition*,
15 Marquardt R.R. and Han Z. eds., pp.129-138) indicate that even though currently available enzymes are beneficial for use as feed additives, new enzymes exhibiting high activity and resistance to heat treatment are also desired; however, they note that enzymes exhibiting these properties are not available.

20 Xylanases of Family 11 (also termed Family G xylanases) have several properties suitable for feed applications due to their small size and high activity. An example of a moderate temperature Family 11 xylanases is TrX, which is obtained from *Trichoderma reesei*. Moderate temperature xylanases are proven feed additive enzymes with temperature and pH optima compatible with the physiological conditions
25 in the digestive system of animals. However, these enzymes can not tolerate the high temperature of the pelleting process and become inactive during this step.

Xylanases from high temperature microorganisms (eg. a thermophile), for example *Thermomonospora fusca* xylanase (termed TfX, also a Family 11 xylanase),
30 have also been considered for feed pelleting. The thermostability of such enzymes is sufficient to tolerate the pelleting temperatures. However, thermophilic xylanases have

- 3 -

optimum activity at high temperatures (70-80°C), and several of these enzymes have a high pH optimum of 7-9. When introduced into the digesting system of an animal, with a physiological temperature of around 40°C (e.g. poultry 43°C, a similar temperature is noted within swine) and pH of 3-5 in the digesta, these enzymes
5 function poorly.

Family 11 xylanases have been modified by protein engineering to improve the properties of these enzymes for industrial applications. These modifications have been directed at increasing the temperature and pH optima, along with the thermostability,
10 of these enzymes for specific applications. For example, US 5,405,769 (WO 94/24270) is directed to site-specific mutagenesis of *Bacillus circulans* xylanase (BcX) for the improvement of the thermostability of this enzyme. The disclosed modifications relate to the formation of intermolecular and intramolecular disulfide bonds within BcX, and these modifications resulted in increased thermostability. For example, an
15 improvement in thermostability of up to 6°C with the addition of a single disulfide bond, and up to 10°C with two disulphide bonds was observed. Other modifications included linking the N- and C- termini which increased thermostability by 6°C, or N-terminal mutations, which increased thermostability by 2°C. However, with all of the above modifications the resultant enzymes were either less active (up to 45% less
20 active), or exhibited an increase in the temperature and pH optima. As such these enzymes are not suitable for feed pelleting applications.

US 5,759,840 also discloses modifications to BcX and *Trichoderma reesei* xylanase (TrX) to increase the thermostability; while at the same time increase the
25 temperature and pH optima of these enzymes. Again, these xylanases would not be suitable for feed pelleting applications.

The above results are in agreement with other reports that note that disulfide bonds are not among the thermostabilization mechanisms employed by thermophilic
30 enzymes (Cowan, D.A., 1995, Essays Biochem. 29:193-207), as the disulfide can be broken into dehydroalanine and thiocysteine at temperatures over 80°C. Therefore, the

SUBSTITUTE SHEET (RULE 26)

- 4 -

enhancement of stability of an enzyme using disulfide bonds is limited to lower temperature ranges. The disulfide bond is thus not recommended to improve the stability of the enzyme at high temperatures (Gupta, M.N., 1991, Biotech. Applied Biochem. 14:1-11; Cowan, D.A., 1995, Essays Biochem. 29:193-207.).

5

None of the above documents address methods for obtaining xylanase enzymes using conventional screening techniques, or by modifying xylanase enzymes, that exhibit the properties of higher temperature tolerance while maintaining optimal performance under conditions of physiological pH and temperature.

10

An improvement in the thermostability of *Trichoderma reesei* xylanase II was reported by Paloheimo et al (Paloheimo, M., Mantyla, A., Vehmaanpera, J., Hakola, S., Lantto, R., Lahtinen, T., Parkkinen, E., Fagerstrom, R. and Suominen, P. 1997, in Carbohydrases from *Trichoderma reesei* and Other Microorganisms p255-264). Of the five mutants characterized, the most improved mutant (glutamic acid-38 TrX) retained 50% of activity at 57°C after 9 min, as compared to 7 min by wide type TrX. Arase et al (Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H., 1993, FEBS Lett. 316:123-127) describes several modifications to improve the thermostability of a *Bacillus pumilis* xylanase (BpX), however only up to 40% of the residual enzymatic activity was maintained following incubation of these enzymes at a temperature of 57°C for 20 min. Even though, in both of these studies the effects of increased thermostability on pH and temperature optima of the enzymes were not determined, these enzymes exhibit inadequate thermostability for feed pelleting applications.

25

In spite of a wide range of experience in screening, testing and modifying xylanase enzymes, there are no reports of xylanases that exhibit the combination of properties required for feed pelleting applications: high thermostability, with optimum activity at physiological pH and temperature. No natural xylanases have been selected, nor has any mutation methodology for the Family 11 xylanases been developed to increase thermostability of xylanase enzymes to, without any change in the temperature

30

- 5 -

and pH optima, and a concomitant loss of the specific activity of the enzyme. Such selected natural xylanases, or xylanases prepared using mutation methodology would offer the advantages of enhancement of feed digestibility and processing in pelleting.

5 The present invention is directed to obtaining xylanase enzymes that exhibit the property of increased thermostability, while maintaining pH and temperature optima that are typically found under physiological conditions.

It is an object of the invention to overcome disadvantages of the prior art.

10

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

15

The present invention relates to thermostable xylanase enzymes. More specifically, the present invention is directed to thermostable xylanase enzymes that exhibit high activity at or near physiological pH and temperature, and the use of these xylanase enzymes in feed pelleting applications.

20

According to the present invention there is provided an isolated xylanase comprising at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C, the isolated xylanase being thermostable. The thermostability is characterized by the isolated xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 70°C in the presence of 40% glycerol. The thermostability may also be characterized by the isolated xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 62.5°C.

25

30 The present invention is also directed to a modified xylanase, comprising at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40

- 6 -

to about 60°C, the modified xylanase being thermostable. This invention also embraces a modified xylanase comprising a basic amino acid at position 162 (TrX numbering), or its equivalent position. The basic amino acid is selected from the group consisting of lysine, arginine and histidine. Preferably the basic amino acid is histidine.

This invention also pertains to the modified xylanase as defined above, wherein the modified xylanase comprises at least one disulfide bridge. Preferably, the modified xylanase comprises one or two disulfide bridges.

The present invention is also directed to a modified xylanase as defined above, wherein the xylanase is a Family 11 xylanase. Furthermore, this invention pertains to a modified xylanase, wherein the Family 11 xylanase is from *Trichoderma*.

The present invention is also directed to the modified xylanase as defined above wherein said xylanase is selected from the group consisting of TrX-162H-DS1, TrX-162H-DS2, and TrX-162H-DS4.

This invention also includes a method of obtaining a xylanase comprising:

- i) selecting an organism that exhibits xylanase activity, obtaining xylanase from the organism;
- ii) determining whether the xylanase exhibits at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C;
- iii) determining whether the xylanase is thermostable; and
- iv) retaining the xylanase that express these properties

Step i) of the above method may also include partially purifying the xylanase.

The present invention also pertains to a method of preparing animal feed, wherein the method comprises applying the isolated xylanase as defined above onto the

- 7 -

animal feed to produce a xylanase-animal feed combination, and heat sterilizing the xylanase-animal feed combination. Preferably, the animal feed is a poultry or swine feed.

5 The present invention is directed to obtaining xylanase enzymes that exhibit pH and temperature optima that are found within the digesta of an animal, while at the same time the xylanase molecule exhibits thermostability and can therefore withstand processes associated with sterilizing and producing pelleted feed. The prior art discloses obtaining thermostable enzymes, either through selection of native enzymes,
10 or through genetic engineering, however, these enzymes do not exhibit physiological pH and temperature optima. The prior art also discloses xylanase enzymes that exhibit optimal enzyme activity at physiological pH and temperature, however, these enzymes are not thermally stable. Furthermore, there is nothing in the prior art to suggest that native xylanase enzymes exist, or that xylanase enzymes may be modified as disclosed
15 herein in order to obtain xylanase enzymes that exhibit high temperature tolerance suitable for feed pelleting, and retain optimum enzymatic activity at or near physiological conditions.

 This summary of the invention does not necessarily describe all necessary
20 features of the invention but that the invention may also reside in a sub-combination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

25 These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows the multiple amino acid sequence alignment among family 11 xylanases. The amino acids common to at least 80% of the Family 11 xylanases
30 listed are indicated in bold. The residues common to all Family 11 xylanases are underlined. *Bacillus pumilus* (Bp); *Clostridium acetobutylicum* P262 XynB

- 8 -

(Ca); *Clostridium stercorarium* (Cs); *Ruminococcus flavefaciens* (Rf);
Trichoderma reesei XynII (Tr2); *Trichoderma viride* (Tv); *Trichoderma*
harzianum (Th); *Schizophyllum commune* Xylanase A (Sc); *Aspergillus niger*
 var. *awamori* (An); *Aspergillus tubigenis* (At); *Trichoderma reesei* XynI (Tr1);
 5 *Streptomyces* sp. No. 36a (Ss); *Streptomyces lividans* Xylanase B (S1B);
Streptomyces lividans Xln C (S1C); *Thermomonospora fusca* TfxA (Tf);
Bacillus circulans (Bc); *Bacillus subtilis* (Bs)

FIGURE 2 shows the synthetic oligonucleotides for the construction of gene sequence
 10 encoding the *Trichoderma* xylanase in the plasmid pTrX (SEQ ID NO:18).

FIGURE 3 shows the effect of incubation time on the residual enzymatic activity of
 mutant TrX, TrX-DS1, TrX-162H, TrX-162H-DS1, and TrX-162H-DS4 at
 62.5°C. The data are normalized to that observed at 0 min.

15 FIGURE 4 shows the effect of temperatures on the residual enzymatic activity of
 several of the modified xylanases of the present invention. Figure 4(a) shows
 the residual enzymatic activity of TrX, TrX-DS1, TrX-162H-DS1, TrX-162H-
 DS2, and TrX-162H-DS4 in sodium citrate buffer in a 30 min incubation.
 20 Figure 4(b) shows the effect of temperatures on the residual enzymatic activity
 of the mutant TrX-DS8. For Figures 4(a) and (b) The data are normalized to
 that observed at 48°C. The T_{50} , which is the incubation temperature allowing
 the maintenance of 50% residual activity after 30 min, was determined for each
 mutant TrX.

25 FIGURE 5 shows the effect of temperatures on the residual enzymatic activity of
 mutant TrX, Trx-DS1 and TrX-162H-DS1 in 40% glycerol in a 30 min
 incubation. The data are normalized to that observed at 50°C.

- 9 -

FIGURE 6 shows the effect of incubation time on the residual enzymatic activity of TrX-162H-DS1 in 40% glycerol at 90°C. The data are normalized to that observed at 0 min.

- 5 **FIGURE 7** shows the effect of temperature on release of xylose in a 30 min hydrolysis of soluble xylan by TrX, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 at pH 4.5. The data are normalized to that observed at the temperature optimum.
- 10 **FIGURE 8** shows the effect of pH on the release of xylose in a 7 min hydrolysis of soluble xylan by TrX, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 at 40°C. The data are normalized to that observed at the pH optimum.

- 10 -

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to thermostable xylanase enzymes and their use as feed additives. More specifically, the present invention is directed to thermostable xylanase enzymes that show good thermostability and exhibit high activity at or near physiological pH and temperature.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

By physiological pH and temperature, it is meant the range in temperature and pH compatible with the digestive system within an animal, for example but not limited to, poultry and swine. For example, a suitable physiological temperature range is from about 35 to about 60°C, more preferably, this range is from about 40 to about 50°C. Similarly, a suitable physiological pH range is from about pH 3.0 to about 7.0, preferably, this range is from about pH 3.5 to about 6.0. The time required for the digestion of feed within the gut of an animal varies from animal to animal. For example, in swine digestion of feed is from about 2 to about 4 hours, while in poultry it is up to about 12 hours.

By high activity at physiological pH and temperature, it is meant that the enzyme exhibits at least 40% of its optimum activity at physiological pH and temperature. The optimum pH and temperature range can be outside the physiological range, provided that the enzyme exhibits at least 40% of its optimum activity within the physiological range, for example from about 40 to about 50°C and pH from about 3.5 to about 6. Examples 4 and 5 describe the determination of a suitable xylanase enzyme that exhibits these properties.

- 11 -

"Thermostable" or "thermostability" as used herein refer to a property of an enzyme. An enzyme is considered to be thermostable if it exhibits at least one of the following properties:

- 5 1) the enzyme exhibits at least 30% of its optimal activity following a pre-incubation step of 30 min at 70°C, 80°C, or 90°C, at pH 5.0, in the presence of a stabilizing agent such as 40% glycerol. Preferably, the enzyme exhibits at least 40% of its optimal activity following a 30 min, 70°C pre-incubation step in glycerol, for example but not limited to, 10 TrX-162H-DS1 (Figure 5);
- 2) the enzyme exhibits 30% of its optimal activity following a pre-incubation step of 30 or 60 min at 62.5°C in the absence of a stabilizer. Preferably, the enzyme exhibits at least 40% of its optimal activity 15 following a 30 min pre-incubation, for example but not limited to, TrX-162H-DS1 and TrX-162H-DS4 (Figure 3);
- 3) the enzyme exhibits at least 30% of its optimal activity following a preincubation step of 30 min at 64°C in the absence of a stabilizer. Preferably, the enzyme exhibits at least 40% of its optimal activity 20 following the 30 min, 64°C pre-incubation step, for example but not limited to, TrX-162H-DS1 and TrX-162H-DS4 (Figure 4); or
- 4) the enzyme exhibits at least 30% of its optimal activity following a preincubation step of 30 min at 68°C in the absence of a stabilizer. Preferably, the enzyme exhibits at least 40% of its optimal activity 25 following the 30 min, 68°C pre-incubation step, for example but not limited to, TrX-162H-DS1 and TrX-162H-DS4 (Figure 4).

SUBSTITUTE SHEET (RULE 26)

- 12 -

In each of the above cases, the optimal activity of the enzyme is determined at an optimal pH and temperature for that enzyme in the presence or absence of stabilizer as required.

5 By "TrX numbering" it is meant the numbering associated with the position of amino acids based on the amino acid sequence of TrX (Xyn II - Table 1; Tr2 - Figure 1). As disclosed below and as is evident upon review of Figure 1, Family 11 xylanases exhibit a substantial degree of sequence homology. Therefore, by aligning the amino acids to optimize the sequence similarity between xylanase enzymes and by using the
10 amino acid numbering of TrX as the basis for numbering, the positions of amino acids within other xylanase enzymes can be determined relative to TrX.

By modified xylanase, it is meant the alteration of a xylanase molecule using techniques that are known to one of skill in the art. These techniques include, but are
15 not limited to, site directed mutagenesis, cassette mutagenesis, synthetic oligonucleotide construction, cloning and other genetic engineering techniques. Alterations of a xylanase enzyme, in order to produce a modified xylanase may also arise as a result of applying techniques directed at inducing mutations within native or genetically engineered xylanases via the addition of known chemical mutagens, UV
20 exposure, or other treatments known to induce mutagenesis within a host organisms that express a xylanase of interest. Such techniques are well known within the art.

Table 1 lists the Family 11 xylanases free of cellulase activity. These enzymes share extensive amino acid sequence similarity and possess amino acids common to
25 Family 11, for example two glutamic acid (E) residues serving as the essential catalytic residues, amino acids 86 and 177 (using TrX numbering). Structural comparisons of several Family 11 xylanases via X-ray crystallography indicates that these Family 11 xylanases of bacterial and fungal origins share the same general molecular structure (see for example US 5,405,769; Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H., 1993, FEBS Lett. 316:123-127). Most of the family 11 xylanases
30 identified so far are mesophilic and have low-molecular mass (20kDa).

TABLE 1: Family 11 xylanases

	Microbe	Xylanase	Ref. in Figure 1	Sequence Listing
5	<i>Aspergillus niger</i>	Xyn A	An	SEQ ID NO: 1
	<i>Aspergillus kawachii</i>	Xyn C		
	<i>Aspergillus tubigenensis</i>	Xyn A	At	SEQ ID NO: 2
	<i>Bacillus circulans</i>	Xyn A	Bc	SEQ ID NO: 3
	<i>Bacillus pumilus</i>	Xyn A	Bp	SEQ ID NO: 4
10	<i>Bacillus subtilis</i>	Xyn A	Bs	SEQ ID NO: 5
	<i>Cellulomonas fimi</i>	Xyn D		
	<i>Chainia spp.</i>	Xyn		
	<i>Clostridium acetobutylicum</i>	Xyn B	Ca	SEQ ID NO: 6
	<i>Clostridium stercorarium</i>	Xyn A	Cs	SEQ ID NO: 7
15	<i>Fibrobacter succinognees</i>	Xyn C		
	<i>Neocallimasterix patriciarum</i>	Xyn A		
	<i>Nocardiopsis dassonvillei</i>	Xyn II		
	<i>Ruminococcus flavefaciens</i>	Xyn A	Rf	SEQ ID NO: 8
	<i>Schizophyllum commune</i>	Xyn	Sc	SEQ ID NO: 9
20	<i>Streptomyces lividans</i>	Xyn B	S1B	SEQ ID NO: 10
	<i>Streptomyces lividans</i>	XynC	S1C	SEQ ID NO: 11
	<i>Streptomyces sp. No. 36a</i>	Xyn	Ss	SEQ ID NO: 12
	<i>Streptomyces thermoviolaceus</i>	XynII		
	<i>Thermomonospora fusca</i>	Xyn A	Tf	SEQ ID NO: 13
25	<i>Trichoderma harzianum</i>	Xyn	Th	SEQ ID NO: 14
	<i>Trichoderma reesei</i>	Xyn I	Tr1	SEQ ID NO: 15
	<i>Trichoderma reesei</i>	Xyn II	Tr2	SEQ ID NO: 16
	<i>Trichoderma viride</i>	Xyn	Tv	SEQ ID NO: 17

- 14 -

It is considered within the scope of the present invention that xylanases, including Family 11 xylanases for example but not limited to *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase I, *Trichoderma viride* xylanase, *Streptomyces lividans* xylanase B and *Streptomyces lividans* xylanase C, may be modified following the general approach and methodology as outlined herein. It is also considered within the scope of the present invention that non-Family 11 xylanases may also be modified following the general principles as described herein in order to obtain a xylanase enzyme that is thermostable and exhibits high activity at physiological pH and temperature.

Furthermore, native xylanases may also be obtained by using standard screening protocols in order to identify enzymes that exhibit the properties of increased thermostability yet maintaining high activity at physiological temperature and pH. Such protocols involve:

- selecting of a desired organism, for example a thermophile;
- extracting or obtaining the xylanase from the organism, and partially purifying the enzyme if desired; and
- characterizing the extracted enzyme to determine whether the enzyme is thermostable, as defined above (in the presence or absence of a stabilizing agent, such as glycerol), determining the enzymes pH and temperature optima, and determining the activity of the enzyme at physiological pH and temperature.

Any enzymes identified using the above protocol that exhibit thermostability and high activity at physiological pH and temperature may be used as animal feeds.

- 15 -

The present invention also relates to modified xylanase enzymes that exhibit increased thermostability while maintaining high activity at physiological pH and temperature. For example, and without wishing to limit the present invention in any manner, a modified *Trichoderma reesei* xylanase (TrX) is disclosed that exhibits

5 increased thermostability while maintaining pH and temperature optima at or near physiological range. Two modifications in the TrX were combined in order to obtain a novel xylanase (TrX-162H-DS1). The first modification includes a double mutation to create two cysteines for the formation of a single disulfide bond. Such a modification has been described for *Bacillus circulans* xylanase (C100/C148; BcX

10 amino acid numbering) in US 5,405,769. However, this mutation bestows only a minor increase in the ability of the enzyme to withstand high temperatures (see TrX-DS1, Figures 3-5) and this modification is not adequate to produce an enzyme capable of surviving high temperatures associated with the pelleting process. When this mutation is combined with a second mutation as per the teaching of this invention,

15 involving the substitution of a basic amino acid such as histidine (H) for glutamine (Q) in position 162, the resultant combination mutant xylanase exhibits the desired properties of thermostability (TrX-162H-DS1; see Figures 5 and 6), and greater than 40% of optimum activity at physiological pH (Figure 8), and temperature (Figure 7).

20 Another mutant xylanase in the present invention, TrX-162H-DS4 differs from TrX-162H-DS1 by possessing an additional disulfide (108/158, that is between positions 108 and 158). This type of double disulfide mutant has previously been described for the xylanase of *Bacillus circulans* (C98/C152, 100/148; BcX amino acid numbering; Wakarchuck et al, 1994 Protein Engineering, 7:1379-1386). The BcX

25 mutant does not comprise an equivalent basic amino acid (e.g. H for Q at position 162) substitution as disclosed herein. The mutant TrX-162H-DS4 shows a dramatic increase of thermostability (see Figure 4(a)), with an increase in the T_{50} of TrX-162H-DS4 of 14°C. This is an improvement over the prior art double disulfide BcX mutant which exhibits an increase in the T_{50} of 10°C, thereby demonstrating the contribution of the

30 Q162H mutation in the disulfide mutants of TrX.

SUBSTITUTE SHEET (RULE 26)

- 16 -

The present invention also pertains to additional mutations that have been found to be effective in producing a xylanase that exhibits thermostability and a desirable pH profile. An example of such mutations may be found in, but are not limited to, TrX-DS8. TrX-DS8 includes the mutations listed for N1-TX13 as disclosed in US 5,759,840, namely N1OH, Y27M and N29L, and also includes N44D, Q125A, I129E, Q162H and a disulfide bond between positions 110 and 154. Trx-DS8 exhibits the property of thermostability (Figure 4(b)), a pH profile paralleling that of TrX-162-DS1, and greater than 40% of optimum activity at physiological pH, and temperature.

Xylanase enzymes comprising the substitution of H for Q at position 162 (termed Q162H) in isolation has been reported in US 5,759,840, however, these mutants exhibited no improvement in thermostability or other properties over natural TrX. However, by combining these two modifications, several novel xylanases (TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4) were obtained with improved thermostability. This property was not observed with either mutation alone. Furthermore, these modified xylanases exhibit high activity at or near physiological temperature and pH. These mutations are also found in Trx-DS8 which also exhibits improved thermostability and high activity at or near physiological conditions.

Following the methods of the present invention novel xylanase enzymes may be obtained that are far more suitable for feed pelleting applications than enzymes currently available. Similar modifications may be made in other Family 11 xylanases, including but not limited to, xylanase enzymes obtained from *Trichoderma*, *Streptomyces* and *Schizophyllum*. However, it is also within the scope of the present invention that other xylanase enzymes, in addition to Family 11 xylanases can be modified as disclosed herein in order to obtain xylanases with that are thermostable and exhibit high activity at physiological pH and temperature. Furthermore, it is within the scope of the present invention that native xylanase enzymes with the properties of thermostability and high activity at physiological pH and temperature may be obtained following screening protocols that select for both thermostability and high activity at physiological pH and temperature.

- 17 -

In use, the formulation of the feed enzyme can improve the enzymes thermostability, as adsorption into feed improves stability as the enzyme is brought into contact with its substrate. Therefore, in determining thermostability of the xylanases of the present invention, xylanases were characterized in the presence and
5 absence of stabilizing agents, for example but not limited to glycerol. Fisk and Simpson (1993) have reported that 40% glycerol enhanced the temperature tolerance of wild type TrX by less than +10°C, however, this is much less stability than the enzymes of the present invention. The combination-mutant xylanases of the present invention can tolerate incubation in buffer at a higher temperature (59-69°C), as
10 compared to natural xylanase (55°C; also see Figure 3 and 4). In the presence of 40% glycerol, the combination mutants can retain a substantial portion of their activity at 70 to 90°C (see figure 5), while the natural xylanase is totally inactivated at these temperatures..

15 One of the modifications to the combination mutant xylanase as proposed herein is the substitution of amino acid 162 (TrX numbering, based on Tr2 in Figure 1; which for TrX is glutamine) with the basic amino acid histidine (termed Q162H). However, it is considered within the scope of the present invention that other amino acids may also be substituted at this position. Preferably the substituted amino acid is basic
20 (positively charged), for example lysine (Q162K) or arginine (Q162R). It has been observed herein that the substitution at the position 162, or its equivalent in other Family 11 xylanases, by a basic amino acid such as histidine can greatly improve the thermostability of a xylanase enzyme that comprises at least one intramolecular disulfide bond. Importantly, it has also been observed herein that this substitution at
25 position 162 not only increases thermostability but also does not significantly change the temperature and pH profiles, and the specific activity of the modified xylanase.

Histidine-162 residue (TrX numbering) in the combination mutant is found in several natural Family 11 xylanases, such as those of *Trichoderma harzianum*,
30 *Aspergillus niger*, var. *awamori*, *Aspergillus tubigensis*, *Thermomonospora fusca*, *Bacillus circulans* and *Bacillus subtilis* in the corresponding position. Similarly,

- 18 -

Clostridium acetobutylicum comprises a lysine at this equivalent position. However, all, of these xylanases, with the exception of the *Thermomonospora fusca* xylanase, are produced by mesophilic hosts and exhibit low thermostability. As a result there is no evidence to suggest any beneficial effect on thermostability by presence of a basic amino acid residue at this position. In the *Thermomonospora fusca* xylanase, the N-terminal sequence (1-29) which is distant from the site of the present invention, has been shown to contribute to thermostability, and there is no evidence to suggest that thermostability may be associated with a histidine at this equivalent position (i.e. TrX 162).

This invention is also directed to xylanases that comprise at least one modification that results in increased thermostability while maintaining high activity at physiological pH and temperature. For example, native *Schizophyllum commune* xylanase has a disulfide bond at positions 110/154 (TrX numbering). However, this enzyme exhibits low thermostability. Therefore, this enzyme can be modified using the methods of the present invention to substitute a basic amino acid, either histidine, arginine or lysine for the naturally occurring leucine at position 200 of *Schizophyllum commune* (which is equivalent to position 162 using TrX numbering; see Figure 1; Sc). Therefore, increased thermostability can be achieved through a one-step modification.

Also considered within the scope of the present invention are combination mutants comprising both an intramolecular disulfide bond and a basic amino acid substitution as outlined above. The intramolecular disulfide bond may arise as a result of a mutation at one or more specific residues, for example (per TrX numbering):

- residues-110/-154, for example, but not limited to TrX-162H-DS1 or Trx-DS8;
- residues-108/-158, for example, but not limited to TrX-162H-DS2; or
- residues-108/-158, -110/-154, for example, but not limited to TrX-162H-DS4.

- 19 -

Also considered within the scope of the present invention are modifications of thermostable xylanases, for example, but not limited to TrX. These modifications maintain the thermostability of the native enzyme, yet alter the pH and temperature optima so that they exhibit high activity at physiological pH and temperature not normally associated with the enzyme.

TABLE 2: Modified xylanases

XYLANASE	DESCRIPTION
wild type TrX	wild type <i>T. reesei</i> xylanase.
TrX-162H	TrX mutant with mutation Q162H.
TrX-DS1	TrX mutant with an intramolecular disulfide bond between positions-110 and 154.
TrX-162H-DS1	TrX mutant with two mutations, (i) a disulfide bond between positions-110 and 154, and (ii) mutation Q162H.
TrX-162H-DS2	TrX mutant with two mutations, (i) an intramolecular disulfide bond between positions-108 and 158, and (ii) mutation Q162H.
TrX-162H-DS4	TrX mutant with two mutations, (i) two intramolecular disulfide bonds at residues-110/154 and residues-108/158, and (ii) mutation Q162H.
TrX-DS8	Trx mutant with i) an intramolecular disulfide bond between positions-110 and 154, and ii) comprising mutations N10H, Y27M, N29L, N44D, Q125A, I129E, and Q162H

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

- 20 -

Examples:**Example 1: Construction of the *Trichoderma reesei* mutant xylanases**

5 Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, oligonucleotide phosphorylation, ligation, transformation and DNA hybridization were performed according to well-established protocols familiar to those skilled in the art (Sung, W. L., Yao, F.-L., Zahab, D. M. and Narang, S. A. (1986) Proc. Natl. Acad. Sci. USA 83:561-565) or as recommended
10 by the manufacturer of the enzymes or kit. The buffer for many enzymes have been supplied as part of a kit or constituted following to the instruction of the manufacturers. Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England BioLabs LTD, Mississauga, Ont. A precursor plasmid pXYbc has previously prepared and published (Sung, W. L., Luk, C. K.,
15 Zahab, D. M. and Wakarchuk, W. (1993) Protein Expression Purif. 4:200-206; US 5,405,769). A commonly used *E. coli* strain, HB101 (clonotech Lab, Palo Alto, CA) was used as transformation and expression host for all gene construct. Birchwood xylan was purchased from Sigma (St. Louis, Mo). Hydroxybenzoic acid hydrazide (HBAH) was purchased from Aldrich. Oligonucleotides were prepared with an Applied
20 Biosystem DNA synthesizer, model 380B. Xylanase assays have been performed in a covered circulating water bath (Haake type F 4391) with a fluctuation of "0.1°C. Temperature of the water bath was confirmed with a thermocouple.

A. Construction of the precursor plasmid pTrX

25

The precursor plasmid pTrX for all subsequent mutations is published (Sung et al, 1995). This plasmid is derived from a pUC119 plasmid with a synthetic nucleotide sequence encoding a *Trichoderma reesei* xylanase inserted (Figure 2). Expression of this xylanase and other mutant xylanases subsequently described are

- 21 -

under the control of the *lac* promoter of the pUC plasmid. The total assembly of the gene required two stages, initially for the (92-190) region, then followed by the (1-92) region. The protocol for the construction of this gene is routine and identical to the standard published procedure for many other genes. It required enzymatic phosphorylation of overlapping synthetic oligonucleotides which encodes xylanase. This was followed by their ligation into a appropriately cut plasmid pUC119.

Initially ten overlapping oligonucleotides:

10	XyTv-101,	SEQ ID NO:28
	XyTv-102,	SEQ ID NO:29
	TrX-103,	SEQ ID NO:30
	XyTv-104,	SEQ ID NO:31
	XyTv-105,	SEQ ID NO:32
15	XyTv-106,	SEQ ID NO:33
	XyTv-107,	SEQ ID NO:34
	TrX-108,	SEQ ID NO:35
	XyTv-109,	SEQ ID NO:22
	XyTv-110,	SEQ ID NO:36

20

encoding the TrX(92-190) sequence (Figure 2), were designed with codon usage frequency imitating that of *E. coli* (Chen et al. 1982). The *Sal*I and *Bgl*II cohesive ends of two terminal oligonucleotides enabled the enzymatic ligation of the ten fragments to the linearized plasmid pXYbc. The ten oligonucleotides (50 pmol, 1 L for each) encoding the TrX(92-190) was phosphorylated in a mixture containing 10X standard kinase buffer (0.4 L), 1mM ATP (4 L), T4 DNA kinase (5 units), and water (3 L). Phosphorylation reaction was carried out for 1 h at 37°C. The solutions were then combined and heated to 70°C for 10 min. After being cooled slowly to room

SUBSTITUTE SHEET (RULE 26)

- 22 -

temperature, the combined solutions were added to a mixture of 4mM ATP (3.5 L), EcoR1-HindIII linearized plasmid pUC119 (0.1 pmol), and T4 DNA ligase (3.5 L) and incubated at 12°C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* HB101 in YT plate (8 g yeast extract, 5 g bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water) containing ampicillin (100 mg/L).

For the preparation of a hybridization probe, one of the oligonucleotide XyTv-110 (10 pmol, 1 L) was phosphorylated ³²P-ATP (10 pmol, 3 L) in T4 DNA kinase (1 L), 10X kinase buffer (1 L), and water (4 L) at 37°C for 1 h.

10

Transformants were selected randomly for hybridization analysis. Colonies were grown on nylon filters on YT plates with ampicillin overnight. They were then denatured with 0.5N NaOH - 1.5M NaCl (10 min) and neutralized with 0.5N Tris-HCl (pH 7.0) - 1.5M NaCl (10 min). After irradiation by UV of 254 nm for 8 min, the filters were washed with 6X SSC - 0.05% Triton X-100 for 30 min. Cell debris was scraped off completely. After another 30 min in fresh solution, the duplicate filters were transferred individually into separate mixtures of 6X SSC - 1% dextran sulphate - 0.05% TritonX-100 - 1X Denhardt's hybridization fluid. The ³²P-labelled probe was added to the filter. After 16 h at 45°C, the filter was washed twice with 6X SSC - 0.05% TritonX-100 at room temperature for 5 min. and then at 65°C for 30 min. Positively hybridized clones with the intermediate plasmid pBcX.TrX were identified by auto-radiographic analysis.

The above protocol, involving enzymatic phosphorylation of synthetic overlapping oligonucleotides and ligation into a linearized plasmid, has again been used in the assembly of the TrX(1-92) region and in the cassette mutagenesis for the subsequent generation of other mutant series described in this invention.

- 23 -

For the assembly of the TrX(1-92) region to complete the full-length *Trichoderma* gene, the intermediate plasmid pBcX.TrX was linearized by NheI and KpnI endonucleases to release the DNA insert for BcX(1-83). With NheI and KpnI cohesive ends, eight overlapping oligonucleotides:

- 5
- | | | |
|----|---------|---------------|
| | TrX-1, | SEQ ID NO:37 |
| | XyTv-2, | SEQ ID NO:38 |
| | TrX-3, | SEQ ID NO:39 |
| | XyTv-4, | SEQ ID NO:40 |
| 10 | XyTv-5, | SEQ ID NO:41 |
| | TrX-6, | SEQ ID NO:42 |
| | XyTv-7, | SEQ ID NO:43 |
| | TrX-8, | SEQ ID NO:44, |

- 15 encoding the published TrX(1-91) sequence were ligated into the linearized plasmid pBcX.TrX (Figure 2), via the protocol described above. The new plasmid pTrX therefore harbored a synthetic TrX gene (SEQ ID NO: 18).

- 20 All mutant xylanases described below have been constructed via the method of cassette mutagenesis as described above. The protocol for the cassette mutagenesis was identical to that for gene assembly fully described above. Such cassette mutagenesis involved (i) enzymatic phosphorylation of overlapping synthetic oligonucleotides, (ii) their ligation with the linearized plasmid, (iii) transformation into the *E. coli* HB101 competent cells, (iv) identification of the mutant transformants via hybridization with
- 25 the labelled oligonucleotide as probe, and (v) confirmation of the mutation through dideoxy nucleotide sequencing.

B. Construction of the plasmid pTrX-DS1

SUBSTITUTE SHEET (RULE 26)

- 24 -

The mutant TrX-DS1 (SEQ ID NO's:54, 55) was identical to TrX with a covalent disulfide bond between residues-110 and 154. This was accomplished through two single mutations, ie. conversion of both residues serine-110 and asparagine-154 to cysteine. Upon expression of the mutant xylanase, these two cysteine residues will form a disulfide bond. The construction of the plasmid pTrX-DS1 was through ligation of the following overlapping phosphorylated oligonucleotides:

TX-110C SEQ ID NO:19,
TX-110C-2 SEQ ID NO:20,
10 TX-103b SEQ ID NO:21,
XyTv-109 SEQ ID NO:22,
TX-108b SEQ ID NO:23,
TX-154C SEQ ID NO:24,
TX-154C-2 SEQ ID NO:25,

15 into KasI/AvrII-linearized plasmid pTrX in a cassette mutagenesis as shown below.

- 25 -

TX-110C-2

101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
 G A T K L G E V T C D G S V Y
 5 5'-GC GCC ACA AAA TTA GGC GAA GTC ACT TGT GAT GGA TCC GTA TAT
 3'-G TGT TTT AAT CCG CTT CAG TGA ACA CTA CCT AGG CAT ATA
 Kasi | TX-110C |

TX-103b

10 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131
 D I Y R T Q R V N Q P S I I G T
 GAT ATC TAC CGT ACC CAA CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC
 CTA TAG ATG GCA TGG GTT GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG

XyTv-109

15 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147
 A T F Y Q Y W S V R R N H R S S
 GCC ACC TTT TAT CAG TAC TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC
 CGG TGG AAA ATA GTC ATG ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG

TX-108b

TX-154C-2

148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
 G S V N T A C H F N A W A Q Q G
 GGT TCG GTT AAT ACT GCA TGC CAC TTT AAT GCC TGG GCA CAG CAA GGG
 25 CCA AGC CAA TTA TGA CGT ACG GTG AAA TTA CGG ACC CGT AGT GTT CCC

SphI

TX-154C

164 165 166 167

L T L G

30 TTA ACC

AAT TGG GAT C

AvrII |

35 C. Construction of the plasmid pTrX-162H-DS1

The mutant TrX-162H-DS1 (SEQ ID NO:56) was identical to TrX-DS1 with a single mutation of glutamine-162 into histidine. The construction of the plasmid pTrX-162D-DS1 was through ligation of oligonucleotides:

40 TX-162H-3 SEQ ID NO: 26, and

TX-162H-4 SEQ ID NO: 27

SUBSTITUTE SHEET (RULE 26)

- 26 -

into SphI/AvrII-linearized plasmid pTrX-DS1 in a cassette mutagenesis, as shown below.

5 TX-162H-3
 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167
 A C H F N A W A Q H G L T L G
 5'-C CAC TTC AAT GCA TGG GCA CAG CAC GGG TTA ACC
GT ACG GTG AAG TTA CGT ACC CGT GTC GTG CCC AAT TGG GAT C-5'
 SphI AvrII
 10 TX-162H-4

D. Construction of the plasmid pTrX-162H-DS2

15 The mutant TrX-162H-DS2 (SEQ ID NO's:57,58) was identical to TrX, but with a covalent disulfide bond between residues 108 and 158, and a mutation glutamine-162 to histidine. The 108/110 disulfide required two single mutations, ie conversion of both residues valine-108 and alanine-158 to cysteine. Upon expression of the mutant xylanase, these two cysteine residues will form a disulfide bond. The construction of the plasmid pTrX-162H-DS2 was through ligation of the following overlapping phosphorylated
 20 oligonucleotides:

TX-108C SEQ ID NO:45,
 TX-108C-2 SEQ ID NO:46,
 TX-103b SEQ ID NO:21,
 XyTv-109 SEQ ID NO:22,
 25 TX-108b SEQ ID NO:23,
 TX-158C-162H SEQ ID NO:47, and
 TX-158C-162H-2 SEQ ID NO:48

into the KasI/AvrII-linearized plasmid pTrX in a cassette mutagenesis as shown below.

- 27 -

TX-108C-2

101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
 G A T K L G E C T S D S S V Y
 5 5-GC GCC ACA AAA TTA GGC GAA TGC ACT AGT GAT GGA TCC GTA TAT
 3'-G TGT TTT AAT CCG CTT ACG TGA TCA CTA CCT AGG CAT ATA
 KasI | TX-108C |

TX-103b

116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131
 D I Y R T Q R V N Q P S I I G T
 GAT ATC TAC CGT ACC CAA CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC
 CTA TAG ATG GCA TGG GTT GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG
 XyTv-109

15

132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147
 A T F Y Q Y W S V R R N H R S S
 20 GCC ACC TTT TAT CAG TAC TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC
 CGG TGG AAA ATA GTC ATG ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG
 TX-108b

25

TX-158C-162H-2

148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
 G S V N T A N H F N C W A Q H G
 GGT TCG GTT AAT ACT GCA AAT CAC TTT AAT TGC TGG GCA CAG CAC GGG
 30 CCA AGC CAA TTA TGA CGT TTA GTG AAA TTA ACG ACC CGT AGT GTG CCC
 TX-158C-162H

35

164 165 166 167
 L T L G
 TTA ACC
 AAT TGG GAT C
 AvrII |

40

SUBSTITUTE SHEET (RULE 26)

- 28 -

E. Construction of the plasmid pTrX-162H-DS4

The mutant TrX-162H-DS4 (SEQ ID NO's:59, 60) was identical to TrX, but with two covalent disulfide bonds 108/158 and 110/154 and a mutation glutamine-162 to histidine. The two disulfides required four single mutations, ie. conversion of the residues
5 valine-108, serine-110, asparagine-154 and alanine-158 to cysteine. Upon expression of the mutant xylanase, these four cysteine residues will form two disulfide bonds. The construction of the plasmid pTrX-162H-DS4 was through ligation of the following overlapping phosphorylated oligonucleotides:

10

TX-108C-110C	SEQ ID NO:49,
TX-108C-110C-2	SEQ ID NO:50,
TX-103b	SEQ ID NO:21,
XyTv-109	SEQ ID NO:22,
15 TX-108b	SEQ ID NO:23,
TX-154C-158C-162H	SEQ ID NO:51 and
TX-154C-158C-162H-2	SEQ ID NO:52

into the KasI/AvrII-linearized plasmid pTrX in a cassette mutagenesis, as shown below.

- 29 -

TX-108C-110C-2

101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
 G A T K L G E C T C D G S V Y
 5 S'GC GCC ACA AAA TTA GGC GAA TGC ACT TGT GAT GGA TCC GTA TAT
 3'-G TGT TTT AAT CCG CTT ACG TGA ACA CTA CCT AGG CAT ATA
 KasI | TX-108C-110C |

TX-103b

10 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131
 D I Y R T Q R V N Q P S I I G T
 GAT ATC TAC CGT ACC CAA CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC
 CTA TAG ATG GCA TGG GTT GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG
 15 XyTv-109

132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147
 A T F Y Q Y W S V R R N H R S S
 20 GCC ACC TTT TAT CAG TAC TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC
 CGG TGG AAA ATA GTC ATG ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG
 TX-108b

TX-154C-158C-162H-2

148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
 G S V N T A C H F N C W A Q H G
 GGT TCG GTT AAT ACT GCA TGC CAC TTT AAT TGC TGG GCA CAG CAC GGG
 30 CCA AGC CAA TTA TGA CGT ACG GTG AAA TTA ACG ACC CGT AGT GTG CCC
 SphI TX-154C-158C-162H

35 164 165 166 167
 L T L G
 TTA ACC
 AAT TGG GAT C

40 AvrII |

45

SUBSTITUTE SHEET (RULE 26)

- 30 -

F. Construction of TrX-DS8

The mutant TrX-DS8 was prepared using analogous methods as those outlined above in Sections A to E for the preparation of modified xylanases. TrX-DS8 incorporates the mutations found in N1-TX13 as disclosed in US 5,759,840. This mutations are N10H, Y27M and N29L. In addition, TrX-DS8 includes the following mutations: N44D, Q125A, I129E, Q162H and a disulfide bond between positions 110 and 154. The construction of the plasmid pTrX-DS8 was through ligation of overlapping phosphorylated oligonucleotides as described above.

10

Trx-DS8 exhibits the property of thermostability (Figure 4a), a pH profile paralleling that of TrX-162-DS1, and greater than 40% of optimum activity at physiological pH, and temperature.

15 **Example 2: Characterization of mutant xylanases**

A. Production of xylanases

The culture condition was identical to the well-established protocol described for other *E. coli*-expressed xylanases. A 5 ml of overnight inoculant in 2YT medium (16 g yeast extract, 10 g bacto-tryptone, 5 g NaCl, 1 L of water) containing ampicillin (100 mg/L) was added to 2YT medium (1 L) with ampicillin. The cultures were grown with shaking (200 rpm) at 37°C. After 16 hr, cells were harvested.

25 B. Purification of different disulfide bond-containing mutant xylanases

Protein samples were prepared from cells by first making an extract of the cells by grinding 10 g of the cell paste with 25 g of alumina powder. After grinding to smooth

- 31 -

mixture, small amounts (5 mL) of ice cold buffer A (10mM sodium acetate, pH 5.5 for BcX mutants) or buffer B (10mM sodium acetate, pH 4.6 for TX mutants) were added and the mixture ground vigorously between additions. The alumina and cell debris were removed by centrifugation of the mixture at 8000 x g for 30 min.

5

The crude extract was heated at 60°C for 15 min and centrifugation to remove a large amount of precipitate. The supernatant was acidified to pH 4.6, frozen at -20°C overnight, thawed and centrifuged to remove more precipitate.

10 After the above pretreatment, the cell extract committed to column chromatography and was pumped onto a 50 mL bed volume, S-Sepharose fast flow, cation exchange column (Kabi-Pharmacia, Canada), equilibrated in buffer A. The xylanase was eluted with a 300 mL linear gradient of 0 to 0.3M NaCl in buffer A at a flow rate of 3 mL/min. The xylanase elutes at 100 to 150 mL of the gradient. The fractions are checked on SDS-PAGE, and those
15 fractions having most of the xylanase were pooled, and concentrated by ultrafiltration using 3000 dalton molecular weight cutoff membranes (Amicon YM3). The concentrated material (5 mL) was then applied to a 1.5 cm x 85 cm TSK-HW50S gel filtration column, equilibrated in 50 mM ammonium acetate pH 6. The xylanase eluted at a volume of 90 to 100 mL. These fractions were analyzed by SDS-PAGE, and the peaks pooled as pure xylanase. The protein
20 was quantified using the extinction co-efficient at 280 nm.

C. Standard assay for the measurement of enzymatic activity

The quantitative assay determined the number of reducing sugar ends generated from
25 soluble xylan. The substrate for this assay was the fraction of birchwood xylan which dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Co.). After removing the insoluble fraction, the supernatant was freeze dried and stored in a desiccator. The measurement of specific activity was performed as follows. Reaction mixtures containing 100 L of 30 mg/mL xylan previously diluted in assay buffer (50 mM sodium citrate, pH 5.5

- 32 -

or the pH optimum of the tested xylanase), 150 L assay buffer, 50 L of enzyme diluted in assay buffer were incubated at 40°C. At various time intervals 50 L portions were removed and the reaction stopped by diluting in 1 mL of 5mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (HBAH) (Lever, 1972, Analytical Biochem 47:273-279). A unit of enzyme activity was defined as that amount generating 1 mol reducing sugar in 1 minute at 40°C.

For the comparison between mutant and the wild type xylanases (TABLE 3), the specific activities of a xylanase was converted to the relative activity which is its calculated in percentage as compared to the specific activity of the natural xylanase.

TABLE 3. Relative activity of TrX xylanases

Xylanase	Relative activity %
natl. TrX	100
TrX	103
TrX-DS1	116
TrX-162H-DS1	102
TrX-162H-DS4	91

The specific activity of the natural TrX (770 U/mg) was normalized to 100%.

As can be seen form Table 3, the specific enzymatic activities of the mutant xylanases at 40°C have not been changed significantly as compared to the natural xylanases.

Example 3: Thermostability of mutant xylanases

This was a test of the tolerance of xylanase to incubation at a set temperature, without any substrate. The xylanase (150 g/mL) in assay buffer (50 mM sodium citrate) was

- 33 -

incubated at a set temperature or set period of time. Aliquots were cooled to room temperature (around 20°C), the residual enzymatic activity of all samples was determined via the HBAH assay at 40°C, as stated in Example 2C.

5 (A) Effect of length of incubation

The effect of the length of incubation on the activity of xylanase samples was determined at 62.5°C at pH 5.5 (Figure 3). Aliquots were removed at 0, 5, 10, 20, 30, 40 and 60 min for the determination of residual activity. The residual enzymatic activity at 0 min was
10 normalized to 100%.

After 5 mins of incubation, the wild type TrX and the Q162H mutant TrX-162H (US 5,759,840) almost lost all residual activity, while the mutant TrX-DS1 with a disulfide bond, retained 60% of its residual activity. However, it retained only 20% of its activity at 20 mins
15 and lost all activity at 40 min. In contrast, the mutant TrX-162H-DS1, with the additional mutation of Q162H, showed superior thermostability by retaining about 87% of its activity at 20 min, 78% at 40 min and 68% at 60 min. The mutant TrX-162H-DS4 with both 108/158 and 110/154 disulfide bonds retained 84% activity after 60 min..

20 (B) Effect of incubation temperatures on the residual activity of mutant TrX.

Thermostability of mutant TrX enzymes was also determined by tolerance of different incubation temperatures. Samples of xylanases were incubated in 50 mM sodium citrate buffer (pH 5.5) at different temperatures (48, 52, 56, 60, 64, 68, 70 and 72°C) for 30 min. The
25 residual enzymatic activity of the samples was determined, with the residual activity at 48°C normalized to 100% (see Figures 4(a) and 4(b)). The T_{50} , which is the incubation temperature allowing the maintenance of 50% residual activity after 30 min, was determined for each mutant TrX.

30 Without wishing to be bound by theory, the higher T_{50} of TrX-162H-DS1 (65°C) versus TrX-DS1 (61°C) demonstrates the enhancement of thermostability by the mutation

- 34 -

Q162H in the disulfide mutants. The double disulfide mutant TrX-162H-DS4 also exhibited high stability with a T_{50} gain of +14°C over the natural TrX. Comparison of T_{50} of TrX-162H-DS1 (65°C) and TrX-162H-DS2 (59°C) indicates that the 110/154 disulfide in TrX-162H-DS1 contributes greater thermostability than the 108/158 disulfide in the latter. TrX-DS8 also exhibited high thermostability, with a T_{50} gain of +16°C when compared to natural TrX.

(C) Effective incubation temperature

In the following example, a model study of the effect of the enzyme formulation on thermostability of the combination mutant was conducted in the presence of an additive, glycerol. The unmodified TrX and the mutant TrX xylanases were incubated for 30 min at 20, 50, 60, 70, 80 and 90°C in a buffer (pH 5.0) with 40% glycerol. The residual activity was determined by the HBAH assay. The residual enzymatic activity at 0 min was normalized to 100% (Figure 5).

At 50°C, all TrX samples retained their enzymatic activity. At 60°C, the wild type TrX retained 75% of its activity while TrX-DS1 and TrX-162H-DS1 retained 80 and 100% respectively (Figure 5). At 70°C, TrX-DS1 and TrX-162H-DS1 maintained 10 and 98% respectively. At 90 min, the latter retained 65% of the residual activity.

(D) Effect of incubation time on the residual activity of TrX-162H-DS1 at 90°C

Sample of TrX-162H-DS1 in 40% glycerol and buffer were incubated in a covered circulating water bath (Haake type F 4391, with a fluctuation of 0.1°C) at 90°C. Temperature of the water bath was confirmed with a thermocouple. Aliquots were removed at 0, 5, 10 and 30 min for assay of residual activity. The residual enzymatic activity at 0 min was normalized to 100%.

At 5, 10 and 30 min, TrX-162H-DS1 retained 90, 85 and 65% of the residual activity respectively (Figure 6).

- 35 -

Example 4: Temperature/activity profile of mutant xylanases

This was a test on the effect on different temperatures to the enzymatic activity of the xylanase in the hydrolysis of soluble xylan. The procedure was identical to the standard assay (Example 2 C) with changes in the incubation temperature and time. The enzymes (1.5 $\mu\text{g/mL}$) and soluble xylanase in 50 mM sodium citrate buffer of pH 4.5 were mixed and incubated in a circulating water bath at different temperatures. After 30 min, the amount of reducing sugars released from xylan was determined by HBAH and was calculated as relative activity, with the value at temperature optimum as 100%.

The effect of temperature on the hydrolysis of xylan was shown in Figure 7. The natural TrX, TrX-DS1, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 enzymes all had the same temperature/activity profile, and the only difference is in the greater activity (80%) in mutant TrX-162H-DS4 as compared to the others (45%) at 60°C. These results indicate that the disulfide mutation, along with the Q162H mutation, has little or no effect on the optimal temperature (50°C) of TrX. In addition, all of the enzymes shown in the figure exhibit at least 40% of their optimum activity from about 40 to about 50°C, which is suitable for feed pelleting applications.

Example 5: pH/activity profile of mutant xylanases

This was a test of the effect of different pH on the enzymatic activity of the xylanase in the hydrolysis of soluble xylan at the approximate physiological temperature of digesta.

The procedure was identical to the standard assay (Example 2 C) with changes in the incubation temperature and time. The *Trichoderma* enzymes natural TrX and mutant TrX (30 $\mu\text{g/mL}$) and soluble xylan in 50 mM sodium citrate buffers of pH 3-8 were incubated together at 40°C for 7 min. The amount of reducing sugars released from xylan was determined by HBAH and was calculated as relative activity, with the value at pH optimum as 100%.

- 36 -

The profile of the effect of pH on the enzymatic activity of TrX, TrX-162H-DS1 and TrX-162H-DS2 (Figure 8) are similar, thus indicating little or no effect of the mutations (disulfide bond formation and Q162H) on the pH optimum. The pH profile for TrX-DS8 was also similar to these modified xylanases (data not shown). All of the enzymes shown in the figure exhibit at least 40% of their optimum activity from about pH 3.5 to about pH 6, which is suitable for feed pelleting applications.

The double disulfide mutant TrX-162H-DS4 differed by showing slightly greater activity at the pH range higher than 6. At the acidic pH of 4-6 TrX, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 maintained at least 75% optimal activity.

All citations listed herein are incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

References

20

Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H. (1993) FEBS Lett. 316:123-127.

25

Beauchemin, K.A., Jones, S.D.M., Rode, L.M., and Sewalt, V.J.H. (1997) Can. J. Animal Sci. 77:645-653.

Beauchemin, K.A. and Rode, L.M. (1997) in Dairy Research Results from the Agriculture and Agri-Food Canada Research Center, Lethbridge, 1E1:1-2.

- 37 -

Bedford, M.R. and Classen, H.L. (1992) in *Xylans and Xylanases*, edited by J. Visser, G. Beldman, M. A. Kusters-van Someren and A. G. J. Voragen, published by Elsevier, Amsterdam, 1992. p361-370.

5

Cowan, D.A. (1995) *Essays Biochem.* 29:193-207.

Fisk, R. S. and Simpson, C. (1993) in *Stability and Stabilization of Enzymes*, edited by W. J. J. van den Tweel, A. Harder and R. M. Buitelaar; published by Elsevier Science Publishers B. V. pp323-328.

10

Gupta, M.N. (1991) *Biotech. Applied Biochem.* 14:1-11.

Irwin, D., Jung, E. D. and Wilson, D. B. (1994) *Appl. Environ. Microbiol.* 60:763-770.

15

Paloheimo, M., Mantyla, A., Vehmaanpera, J., Hakola, S., Lantto, R., Lahtinen, T., Parkkinen, E., Fagerstrom, R. and Suominen, P. (1997) in *Carbohydrases from Trichoderma reesei and Other Microorganisms* p255-264.

20

Sung, W. L., Yao, F.-L., Zahab, D. M. and Narang, S. A. (1986) *Proc. Natl. Acad. Sci. USA* 83:561-565.

Sung, W. L., Luk, C. K., Zahab, D. M. and Wakarchuk, W. (1993) *Protein Expression Purif.* 4:200-206.

25

Sung, W. L., Luk, C. K., Chan, B., Wakarchuk, W., Yaguchi, M., Campbell, R., Willick, G., Ishikawa, K. and Zabab, D. M. (1995) *Biochem. Cell. Biol.* 73:253-259.

Torronen, A. and Rouvinen, J. (1995) *Biochemistry* 34:847-856.

30

- 38 -

Viveros, A., Brenes, A., Pizarro, M. and Castano, M. (1994) Animal Feed Sci. Technol.
48:237-251.

Wakarchuck W. W., Sung, W. L., Campbell, R. L., Cunningham, A., Watson, D. C. and
5 Yaguchi, M. (1994) Protein Engineering 7:1379-1386.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated xylanase characterized in exhibiting at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 50°C, said xylanase being thermostable.
2. The isolated xylanase of claim 1 wherein said xylanase is characterized in exhibiting at least 40% of optimal activity from about 40 to about 60°C.
3. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 70°C in the presence of 40% glycerol.
4. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 80°C in the presence of 40% glycerol.
5. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 90°C in the presence of 40% glycerol.
6. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 60 minutes at 62.5°C.
7. The isolated xylanase of claim 5 wherein said thermostability is determined in the absence of stabilizer.
8. The isolated xylanase of claim 6 wherein said thermostability is determined in the absence of stabilizer.

- 40 -

9. The isolated xylanase of claim 2, wherein said xylanase is a modified xylanase.
10. The isolated xylanase of claim 9, wherein said xylanase is a Family 11 xylanase.
11. The isolated xylanase of claim 10, wherein said Family 11 xylanase is a *Trichoderma* xylanase.
12. A modified xylanase comprising a basic amino acid at position 162 (TrX numbering) or its equivalent, exhibiting at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C, said modified xylanase being thermostable.
13. The modified xylanase of claim 12, wherein said basic amino acid is selected from the group consisting of lysine, arginine and histidine.
14. The modified xylanase of claim 13, wherein said basic amino acid is histidine.
15. The modified xylanase of claim 9 comprising at least one disulfide bridge.
16. The modified xylanase of claim 9 comprising two disulfide bridges.
17. The modified xylanase of claim 9 comprising a basic amino acid at position 162 (TrX numbering) or its equivalent position, and at least one disulfide bridge.
18. The modified xylanase of claim 9 selected from the group consisting of TrX-162H-DS1, TrX-162H-DS2, TrX-162H-DS4, and TrX-DS8.
19. The modified xylanase of claim 18, wherein said xylanase is TrX-162H-DS1.
20. The modified xylanase of claim 18, wherein said xylanase is TrX-162H-DS2.
21. The modified xylanase of claim 18, wherein said xylanase is TrX-162H-DS4.

- 41 -

22. The modified xylanase of claim 18, wherein said xylanase is TrX-DS8.
23. A method of obtaining xylanase comprising:
- i) selecting an organism that expresses xylanase activity, and obtaining said xylanase from said organism;
 - ii) determining whether said xylanase exhibits at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C; and
 - iii) determining whether said xylanase is thermostable; and
 - iv) retaining said xylanase that express these properties.
24. The method of claim 24, wherein step i) includes partially purifying said xylanase.
25. A method of preparing animal feed comprising applying the isolated xylanase of claim 1 onto said animal feed to produce a xylanase-animal feed combination, and heat sterilizing said xylanase-animal feed combination.
26. The method of claim 25, wherein said animal feed is a poultry or swine feed.
27. A method of preparing animal feed comprising, applying the xylanase obtained from step iv) of claim 23 onto said animal feed to produce a xylanase-animal feed combination, and heat sterilizing said xylanase-animal feed combination.
28. The method of claim 27, wherein said animal feed is a poultry or swine feed.

1/12

Ca	23			S	AFNTQAAP	31
Cs	1			G		1
Bp	1	RTITNEMGN	HSGYDYELWK DYGNT-SMTL NNGGAFSAGW	N--NIGNA		45
Ca	32	KTITSNEIGV	NGGYDYELWK DYGNT-SMTL KNNGAFSCQW	S--NIGNA		76
Cs	2	RIIYDNETGT	HGGYDYELWK DYGNT-IMEL NDGGTFSCQW	S--NIGNA		46
Rf	1	SAADQQTRGN	VGGYDYEMWN QNGQOQASMN PGAGSFTCSW	S--NIENF		46
Tr2	1	QTIQPGTGY	NNGYFYSYWN DGHGGVTTYTN GPGGQFSVNW	S--NSGNF		45
Tv	1	QTIQPGTGF	NNGYFYSYWN DGHGGVTTYTN GPGGQFSVNW	S--NSGNF		45
Th	1	QTIGPGTGY	SNGYFYSYWN DGHAGVTTYTN GGGGSFTVNW	S--NSGNF		45
Sc	1	SGTPSSSTGT	DGGYFYSWWT DGAGDATYQN NGGGSYTLTW	SG--NNGNL		46
An	1		S AGINYVQNYN QNLGDFTY-D ESAGTFSMYW	EDGVSSDF		38
AT	1		S AGINYVQNYN QNLGDFTY-D ESAGTFSMYW	EDGVSSDF		38
Tr1	1		ASINYDQNYQ TGG-QVSY- PSNTGFSVNW	N--TQDDF		34
Ss	1	ATTIT-NETGY	D-GMYYSFWT DGGGSVSMTL NGGGSYSTRW	T--NCGNF		45
SlB	1	DTVVTNQEGT	NNGYFYSFWT DSQGTVMNM GSGGQYSTSW	R--NTGNF		47
SlC	1	ATTITNTQTGT	D-GMYYSFWT DGGGSVSMTL NGGGSYSTQW	T--NCGNF		46
Tf	1	AVTSNETGY	HDGYFYSFWT DAPGTVMEL GPGGNYSTSW	R--NTGNF		45
Bc	1		ASTDYWQNW DGGGIVNAVN GSGGNYSVNW	S--NTGNF		36
Bs	1		ASTDYWQNW DGGGIVNAVN GSGGNYSVNW	S--NTGNF		36
Bp	46	LFRK-GKKFD	ST-RTHHQLG NISINYNASF N-PSGNBYLC	VYGWTSNP		90
Ca	77	LFRK-GKKFN	DT-QTYQLG NISVNYNCNY Q-PYGNSYLC	VYGWTSNP		121
Cs	47	LFRK-GRKFN	SD-KTYQELG DIWVEYGCY N-PNGNSYLC	VYGWTRNP		91
Rf	47	LARM-GKNYD	SQKKNYKAEQ NIVLTYDVEY T-PRGNSYMC	VYGWTRNP		92
Tr2	46	VGGK-GWQPG	TKNKV-----INFS-GS YNPNGNSYLS	VYGWSRNP		83
Tv	46	VGGK-GWQPG	TKNKV-----INFS-GS YNPNGNSYLS	VYGWSRNP		83
Th	46	VGGK-GWQPG	TKNKV-----INFS-GS YNPNGNSYLS	IYGWSRNP		83
Sc	47	VGGK-GWNPB	AASRS-----ISYS-GT YQPNGNSYLS	VYGWTRSS		84
An	39	VVGL-GWTTG	SSNA-----ITYSAEY SASGSSSYLA	VYGWVNYP		76
At	39	VVGLGGWTTG	SSNA-----ITYSAEY SASGSASYLA	VYGWVNYP		77
Tr1	35	VVGW-GWTTG	SSAP-----INFGGSF SVNSGTGLLS	VYGWSTNP		72
Ss	46	VAGK-GWANG	GR-RT-----VRYT-GW FNPNGNGYGC	LYGWTSNP		82
SlB	48	VAGK-GWANG	GR-RT-----VQYS-GS FNPNGNAYLA	LYGWTSNP		84
SlC	47	VAGK-GWSTG	DGN-----VRYN-GY FNPVNGNGYGC	LYGWTSNP		82
Tf	46	VAGK-GWATG	GR-RT-----VTYS-AS FNPNGNAYLT	LYGWTRNP		82
Bc	37	VVGK-GWTTG	SPFRT-----INYNAGV WAPNGNGYLT	LYGWTRSP		75
Bs	37	VVGK-GWTTG	SPFRT-----INYNAGV WAPNGNGYLT	LYGWTRSP		75

FIGURE 1

2/12

Bp 91 LAEYYIVDSW GTYR-PT--G AYKGSFYADG GTYDIYETTR VNQPSIIG 135
 Ca 122 LVEYYVIDSW GSWRPP--GG TSKGTITVDG GIYDIYETTR INQPSIQG 167
 Cs 92 LVEYYIVESW GSWRPP--GA TPKGTITQWMAGTYEYIETTR VNQPSIDG 138
 Rf 93 LMEYYIVEGW GDWRPPGNDG EVKGTVSANG NTYDIRKTMR VNQPSLDG 140
 Tr2 84 LIEYYIVENF GTYN-PSTGA TKLGEVTS DG SVYDIYRTOR VNQPSIIG 130
 Tv 84 LIEYYIVENF GTYN-PSTGA TKLGEVTS DG SVYDIYRTOR VNQPSIIG 130
 Th 84 LIEYYIVENF GTYN-PSTGA TKLGEVTS DG SVYDIYRTOR VNQPSIIG 130
 Sc 85 LIEYYIVESY GSYD-PSSAA SHKGSVTCNG ATYDILSTWR VNAPSIDG 131
 An 77 GA EYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR INEPSITG 123
 At 78 QA EYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR INEPSITG 124
 Tr1 73 LVEYYIMEDN HNY--PAQ-G TVKGTVTS DG ATYTIWENTR VNEPSIQG 117
 Ss 83 LVEYYIVDNW GSYR-PT--G ETRGTVHSDG GTYDIYKTTR VNAPSVEA 127
 SlB 85 LVEYYIVDNW GTYR-PT--G EYKGTVTS DG GTYDIYKTTR VNKPSVEG 129
 SlC 83 LVEYYIVDNW GSYR-PT--G TYKGTVSS DG GTYDIYQTTR VNAPSVEG 127
 Tf 83 LVEYYIVESW GTYR-PT--G TYMGTVTTDG GTYDIYKTTR VNAPSIEG 127
 Bc 76 LIEYYVVD SW GTYR-PT--G TYKGTVKSDG GTYDIYTTTR VNAPSIDG 120
 Bs 76 LIEYYVVD SW GTYR-PT--G TYKGTVKSDG GTYDIYTTTR VNAPSIDG 120

Bp 136 -IATFKQYWS VRQTKRTS-- -----GTVS VSAHFRKWES LGMPM-GK 174
 Ca 168 -NTTFKQYWS VRRTKRTS-- -----GTIS VSKHFAAWES KGMP-L-GK 206
 Cs 139 -TATFQYQYWS VRTSKRTS-- -----GTIS VTEHFKQWER MGMRM-GK 177
 Rf 141 -TATFQYQYWS VRQTS GSANN QTNMKG TID VSKHFD A WS AGLDMSGT 187
 Tr2 131 -TATFQYQYWS VRNRHR-S-S -----GSVN TANHFNAWAQ QGLTL-GT 168
 Tv 131 -TATFQYQYWS VRNRHR-S-S -----GSVN TANHFNAWAQ QGLTL-GT 168
 Th 131 -TATFQYQYWS VRNRHR-S-S -----GSVN TANHFNAWAH HGLTL-GT 168
 Sc 132 -TQTFEQFWS VRNPKKAPGG SIS---GTVD VQCHFD A WK LGMNLGSE 175
 An 124 -TSTFTQYFS VRESTRTS-- -----GTVT VANHFNFWAQ HGFN-SD 162
 At 125 -TSTFTQYFS VRESTRTS-- -----GTVT VANHFNFWAH HGFHN-SD 163
 Tr1 118 -TATFNQYIS VRNSPR-T-S -----GTVT VQNHFN-WAS LGLHLGQM 155
 Ss 128 -PAAFDQYWS VRQSKVT--S -----GTIT TGNHFD A WAR AGMNMGNF 168
 SlB 130 TR-TFDQYWS VRQSKR-TG- -----GTIT TGNHFD A WAR AGMPLGNF 168
 SlC 128 TK-TFQYQYWS VRQSKVTSGS -----GTIT TGNHFD A WAR AGMNMGQF 168
 Tf 128 TR-TFDQYWS VRQSKRTS-- -----GTIT AGNHFD A WAR HGMHLGTH 166
 Bc 121 DR TTFQYWS VRQSKRPTGS N-----ATIT FTNHVNAWKS HGMNLGSN 163
 Bs 121 DR TTFQYWS VRQSKRPTGS N-----ATIT FSNHVNAWKS HGMNLGSN 163

FIGURE 1 (CONT'D)

SUBSTITUTE SHEET (RULE 26)

3/12

Bp	174	MYETAFTVEG	YQSSGSANVM	TNQLFIGN	201
Ca	207	MHETAFNIEG	YQSSGKADVN	SMSINIGK	233
Cs	178	MYEVALTVEG	YQSSGYANVY	KNEIRIGANP....	
Rf	188	LYEVSLNIEG	YRSNGSANVK	SVSV	211
Tr2	169	MDYQIVAVEG	YFSSGSASI-	TVS	190
Tv	169	MDYQIVAVEG	YFSSGSASI-	TVS	190
Th	169	MDYQIVAVEG	YFSSGSASI-	TVS	190
Sc	176	HNYQIVATEG	YQSSGTATI-	TVT	197
An	163	FNYQVMAVEA	WSGAGSASV-	TISS	184
At	164	FNYQVVAVEA	WSGAGSAAV-	TISS	185
Tr1	156	MNYQVVAVEG	WGGSGSASQ-	SVSN	178
Ss	167	RYYMINATEG	YQSSGSSTI-	TVSG	189
SlB	169	SYYMINATEG	YQSSGTSSI-	NVGG.....	
SlC	169	RYYMINATEG	YQSSGSSNI-	TVSG	191
Tf	167	D-YMIMATEG	YQSSGSSNVT	LGTS.....	
Bc	164	WAYQVMATEG	YQSSGSSNV-	TVW	185
Bs	164	WAYQVMATEG	YQSSGSSNV-	TVW	185

Bp	<i>Bacillus pumilus</i>
Ca	<i>Clostridium acetobutylicum</i> P262 XynB
Cs	<i>Clostridium stercorearium</i> xynA
Rf	<i>Ruminococcus flavefaciens</i>
Tr2	<i>Trichoderma reesei</i> XYN II
Tv	<i>Trichoderma viride</i>
Th	<i>Trichoderma harzianum</i>
Sc	<i>Schizophyllum commune</i> Xylanase A
An	<i>Aspergillus niger</i> , var. <i>awamori</i>
At	<i>Aspergillus tubigenensis</i>
Tr1	<i>Trichoderma reesei</i> XYN I
Ss	<i>Streptomyces</i> sp. 36a
SlB	<i>Streptomyces lividans</i> Xln B
SlC	<i>Streptomyces lividans</i> Xln C
Tf	<i>Thermomonospora fusca</i> TfxA
Bc	<i>Bacillus circulans</i>
Bs	<i>Bacillus subtilis</i>

FIGURE 1 (CONT'D)

4/12

st

5'-CT AGC TAA GGA GG CTG CAG ATG
 G ATT CCT CC GAC GTC TAC
 NheI | PstI

TrX-1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Q	T	I	Q	P	G	T	G	Y	N	N	G	Y	F	Y	S
CAA	ACA	ATA	CAA	CCA	GGA	ACC	GGT	TAC	AAC	AAC	GGT	TAC	TTT	TAC	AGC
GTT	TGT	TAT	GTT	GGT	CCT	TGG	CCA	ATG	TTG	TTG	CCA	ATG	AAA	ATG	TCG

TrX-8 AgeI |

XyTv-2

17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Y	W	N	D	G	H	G	G	V	T	Y	T	N	G	P	G
TAT	TGG	AAC	GAT	GGC	CAT	GGT	GGT	GTT	ACC	TAT	ACA	AAC	GGG	CCC	GGA
ATA	ACC	TTG	CTA	CCG	GTA	CCA	CCA	CAA	TGG	ATA	TGT	TTG	CCC	GGG	CCT

NcoI XyTv-7 ApaI

33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
G	Q	F	S	V	N	W	S	N	S	G	N	F	V	G	G
GGC	CAA	TTT	AGC	GTC	AAT	TGG	TCT	AAC	TCC	GGA	AAC	TTC	GTA	GGT	GGA
CCG	GTT	AAA	TCG	CAG	TTA	ACC	AGA	TTG	AGG	CCT	TTG	AAG	CAT	CCA	CCT

MunI | BspEI

TrX-3

49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
K	G	W	Q	P	G	T	K	N	K	V	I	N	F	S	G
AAA	GGT	TGG	CAA	CCC	GGG	ACC	AAA	AAT	AAG	GTG	ATC	AAC	TTC	TCT	GGA
TTT	CCA	ACC	GTT	GGG	CCC	TGG	TTT	TTA	TTC	CAC	TAG	TTG	AAG	AGA	CCT

XmaI TrX-6

65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
S	Y	N	P	N	G	N	S	Y	L	S	V	Y	G	W	S
TCT	TAT	AAT	CCG	AAT	GGG	AAT	TCA	TAC	TTA	AGC	GTC	TAT	GGC	TGG	TCT
AGA	ATA	TTA	GGC	TTA	CCC	TTA	AGT	ATG	AAT	TCG	CAG	ATA	CCG	ACC	AGA

| EcoRI AflII

XyTv-4

81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
R	N	P	L	I	E	Y	Y	I	V	E	N	F	G	T
AGA	AAC	CCA	CTG	ATT	GAA	TAT	TAC	ATT	GTC	GAA	AAT	TTC	GGT	AC
TCT	TTG	GGT	GAC	TAA	CTT	ATA	ATG	TAA	CAG	CTT	TTA	AAG	C	

Xba I XyTv-5 | KpnI

FIG. 2

SUBSTITUTE SHEET (RULE 26)

5/12

|
 XyTv-101
 92 93 94 95 96 97 98 99 100 101 102 103 104 105
 V D N F G T Y N P S T G A T K L
 TC GAC AAT TTC GGT ACC TAC AAT CCG AGT ACC GGC GCC ACA AAA TTA
 G TTA AAG CCA TGG ATG TTA GGC TCA TGG CCG CGG TGT TTT AAT
 SalI | KpnI XyTv-110 KasiI/NarI

|
 XyTv-102
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121
 G E V T S D G S V Y D I Y R T Q
 GGC GAA GTC ACT AGT GAT GGA TCC GTA TAT GAT ATC TAC CGT ACC CAA
 CCG CTT CAG TGA TCA CTA CCT AGG CAT ATA CTA TAG ATG GCA TGG GTT
 SpeI BamHI | XyTv-109

|
 TrX-103
 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137
 R V N Q P S I I G T A T F Y Q Y
 CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC GCC ACC TTT TAT CAG TAC
 GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG CCG TGG AAA ATA GTC ATG
 MluI ClaI |

|
 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153
 W S V R R N H R S S G S V N T A
 TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC GGT TCG GTT AAT ACT GCG
 ACC TCA CAA TCT GCA TTA GTA GGT TCG AGG CCA AGC CAA TTA TGA CGC
 TrX-108 SacI |

|
 XyTv-104
 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169
 N H F N A W A Q Q G L T L G T M
 AAT CAC TTT AAT GCA TGG GCA CAG CAA GGG TTA ACC CTA GGT ACA ATG
 TTA GTG AAA TTA CGT ACC CGT GTC GTT CCC AAT TGG GAT CCA TGT TAC
 NsiI XyTv-107 AvrII

|
 XyTv-105
 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185
 D Y Q I V A V E G Y F S S G S A
 GAT TAT CAA ATC GTA GCG GTG GAA GGC TAC TTC TCG AGT GGT TCC GCT
 CTA ATA GTT TAG CAT CGC CAC CTT CCG ATG AAG AGC TCA CCA AGG CGA
 | XyTv-106 XhoI

|
 186 187 188 189 190
 S I T V S
 AGT ATT ACA GTG AGC TAA A
 TCA TAA TGT CAC TCG ATT TCT AG
 BglII |

FIG. 2 (CONT'D)

6/12

Thermostability of mutant Trx

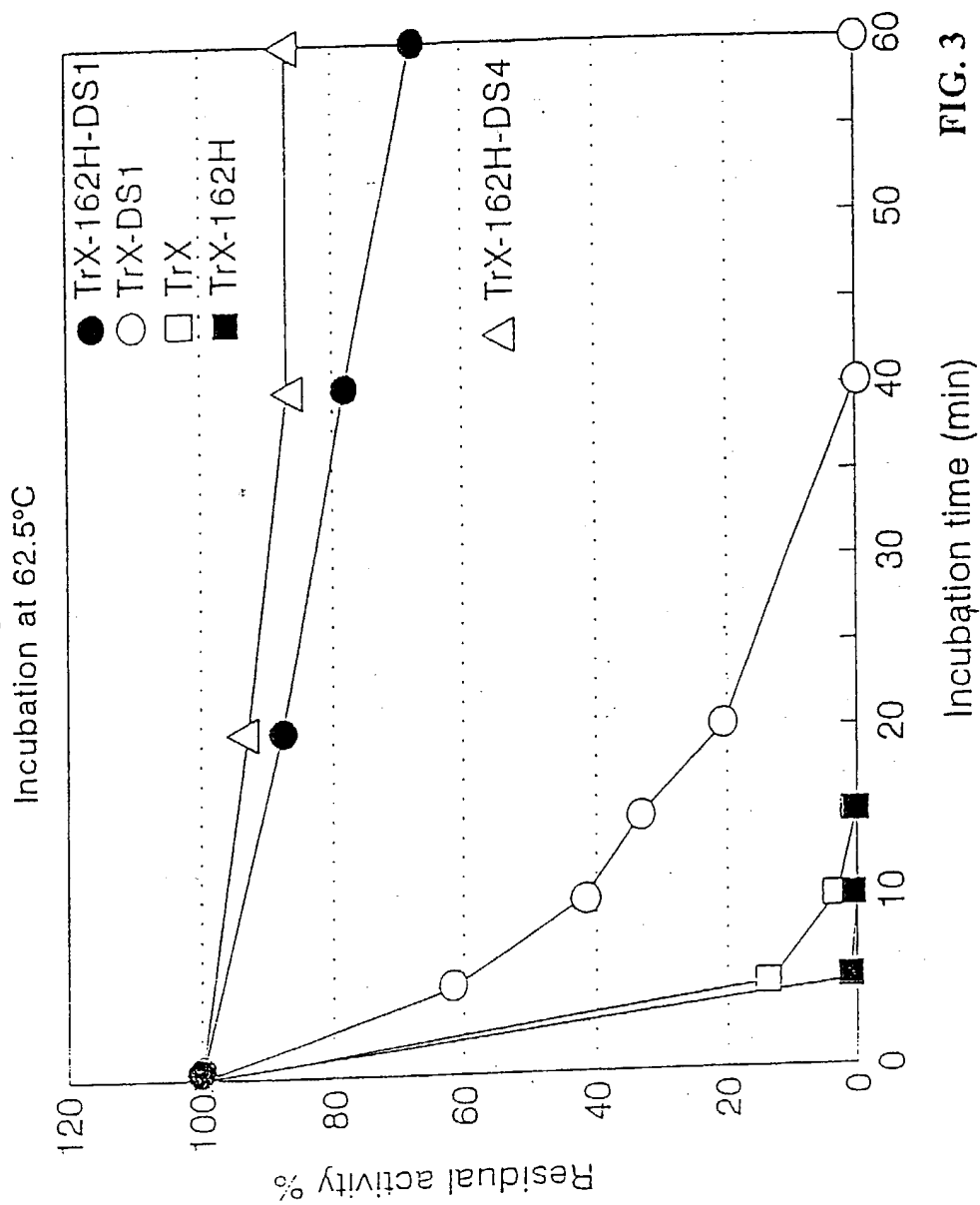


FIG. 3

SUBSTITUTE SHEET (RULE 26)

7/12

Thermostability of mutant TrX

Incubation 30 min. at pH 5.5

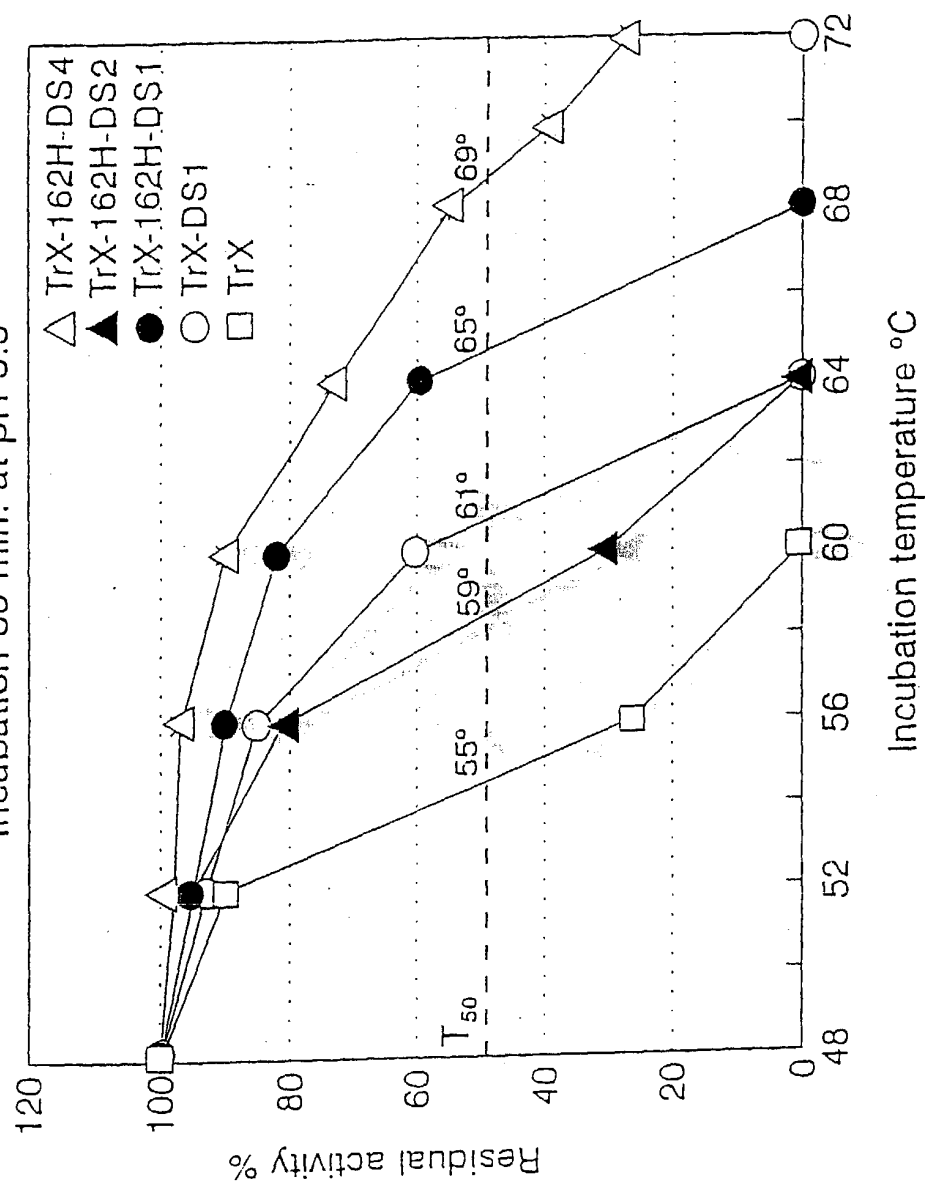


FIG. 4A

8/12

Thermostability of mutant TrX

Incubation 30 min. at pH 5.5

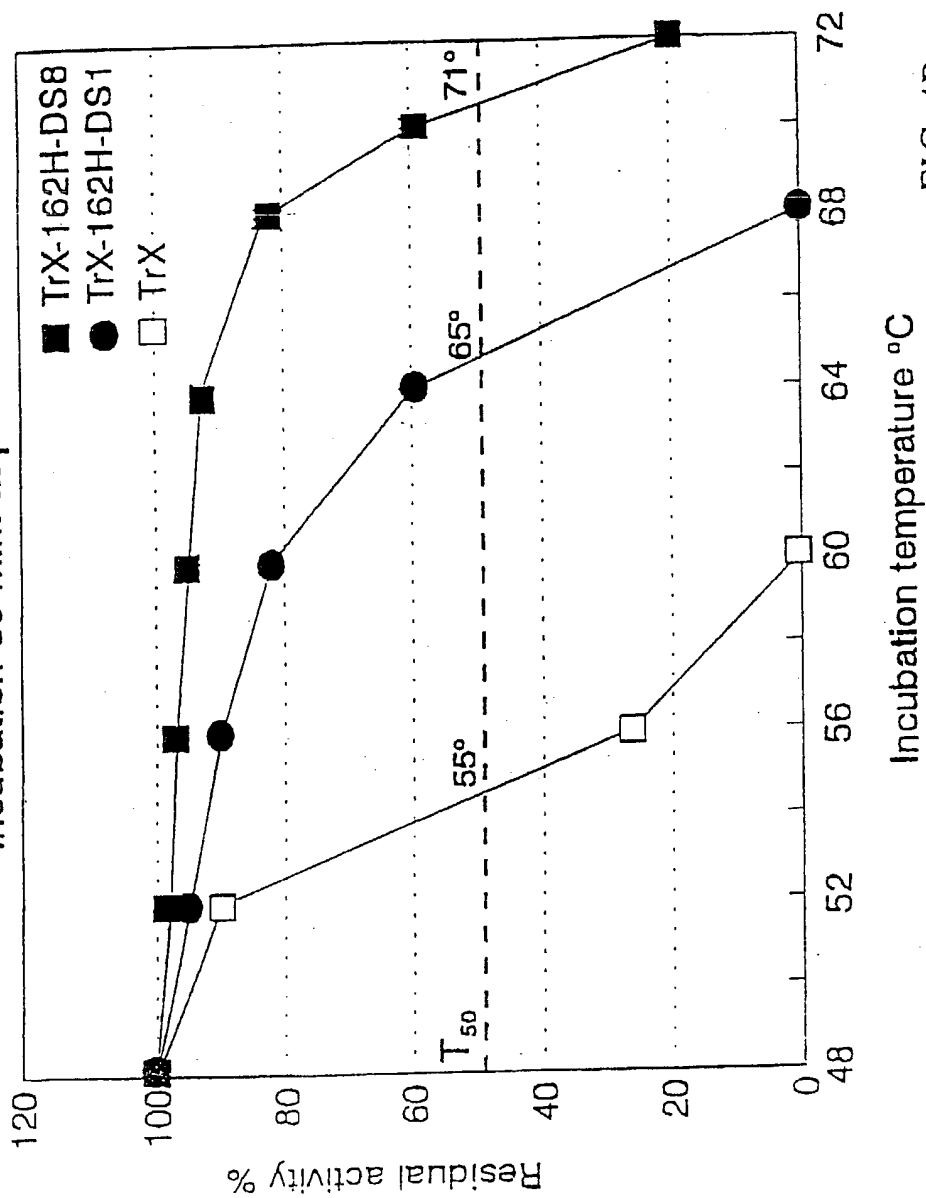


FIG. 4B

9/12

Thermostability of mutant TrX incubation in 40% glycerol, 30 min

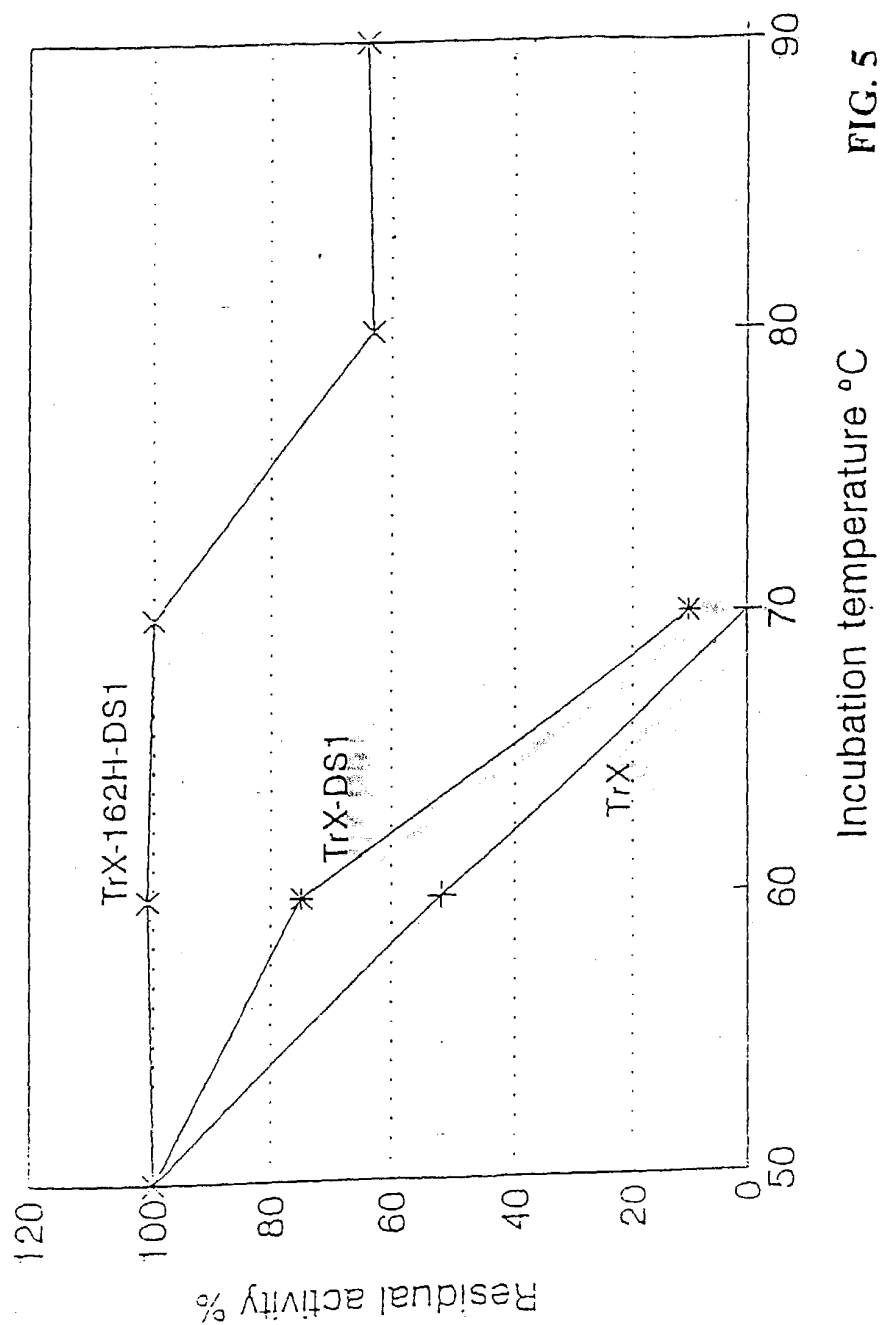


FIG. 5

10/12

Thermostability of TrX-162H-DS1 at 90°C

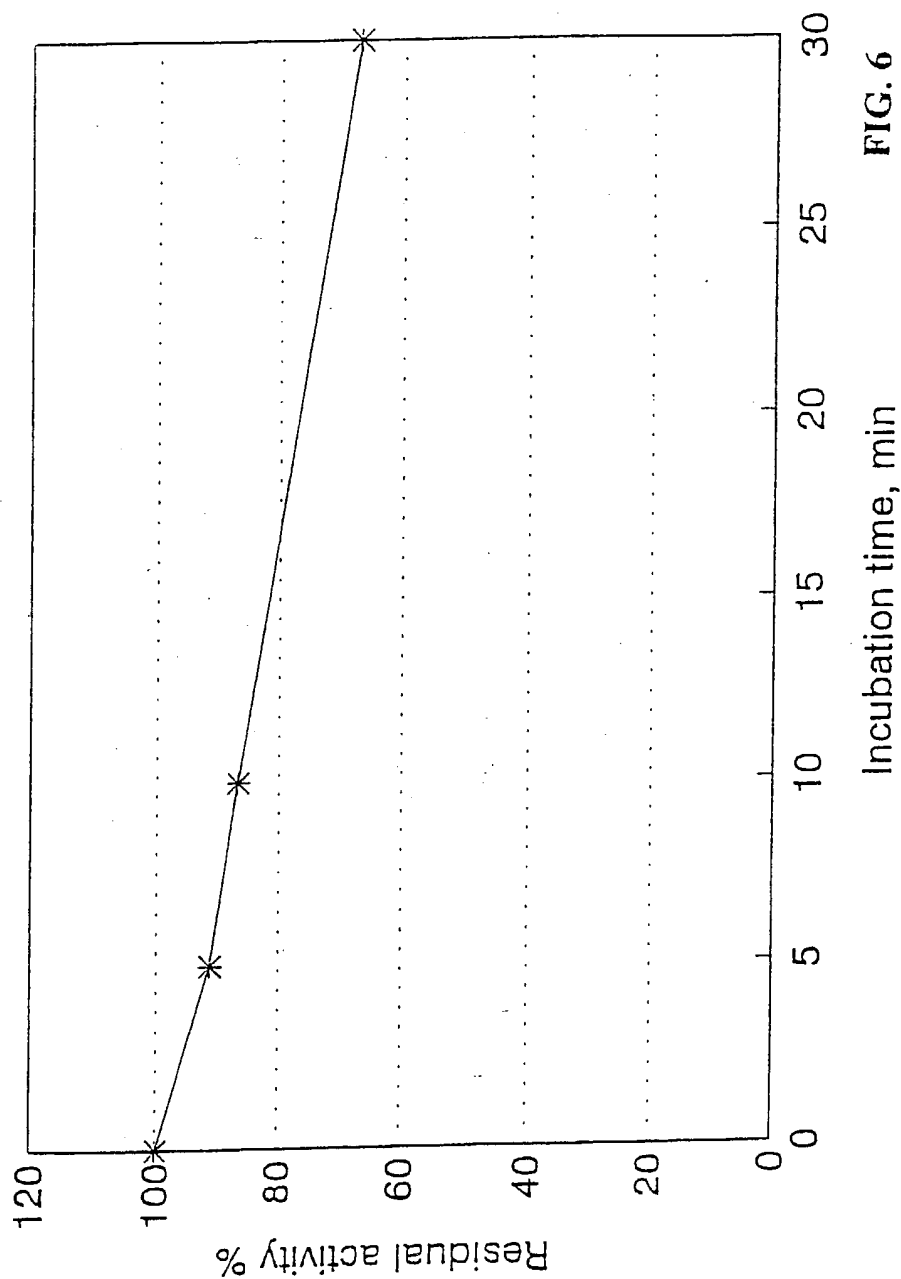
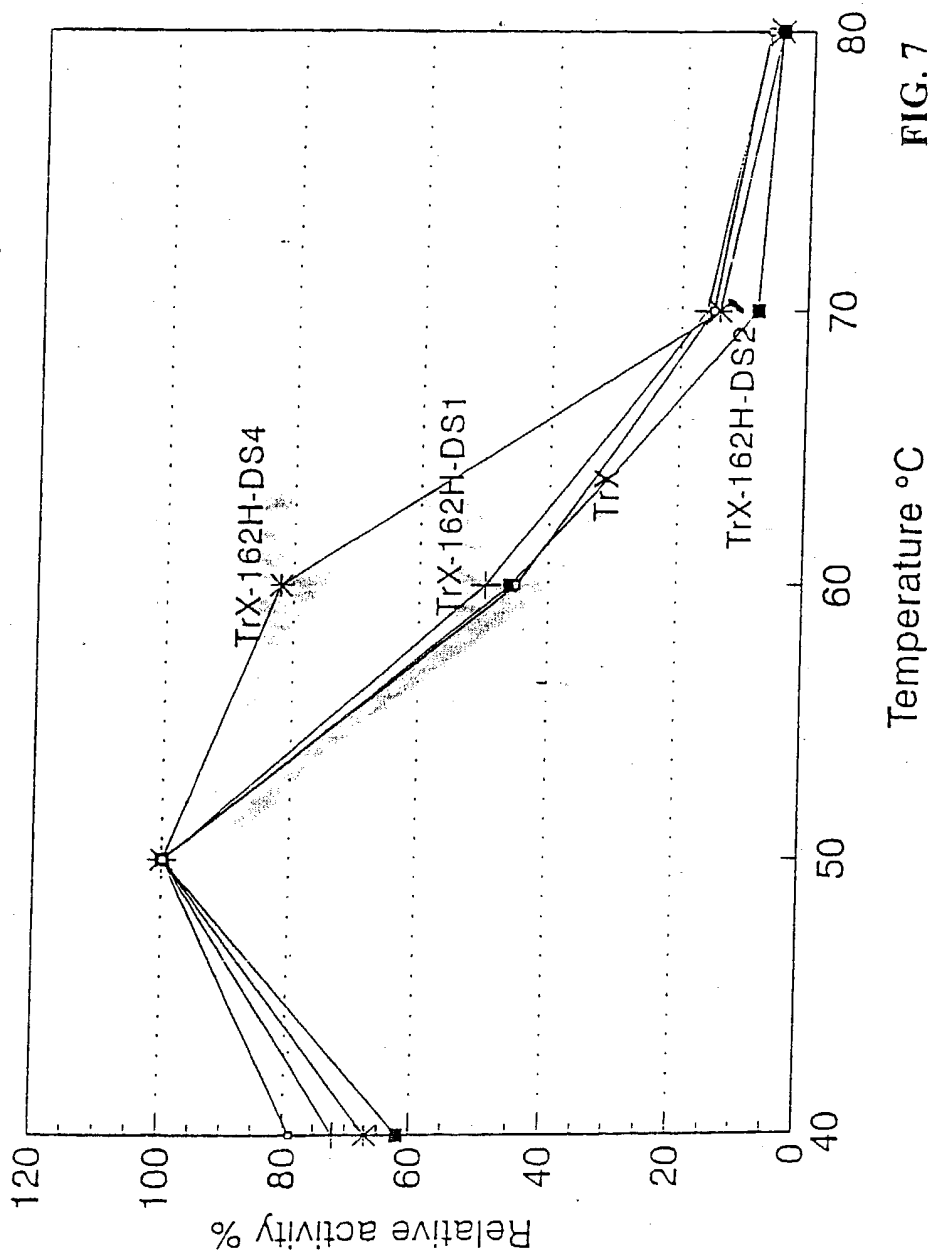


FIG. 6

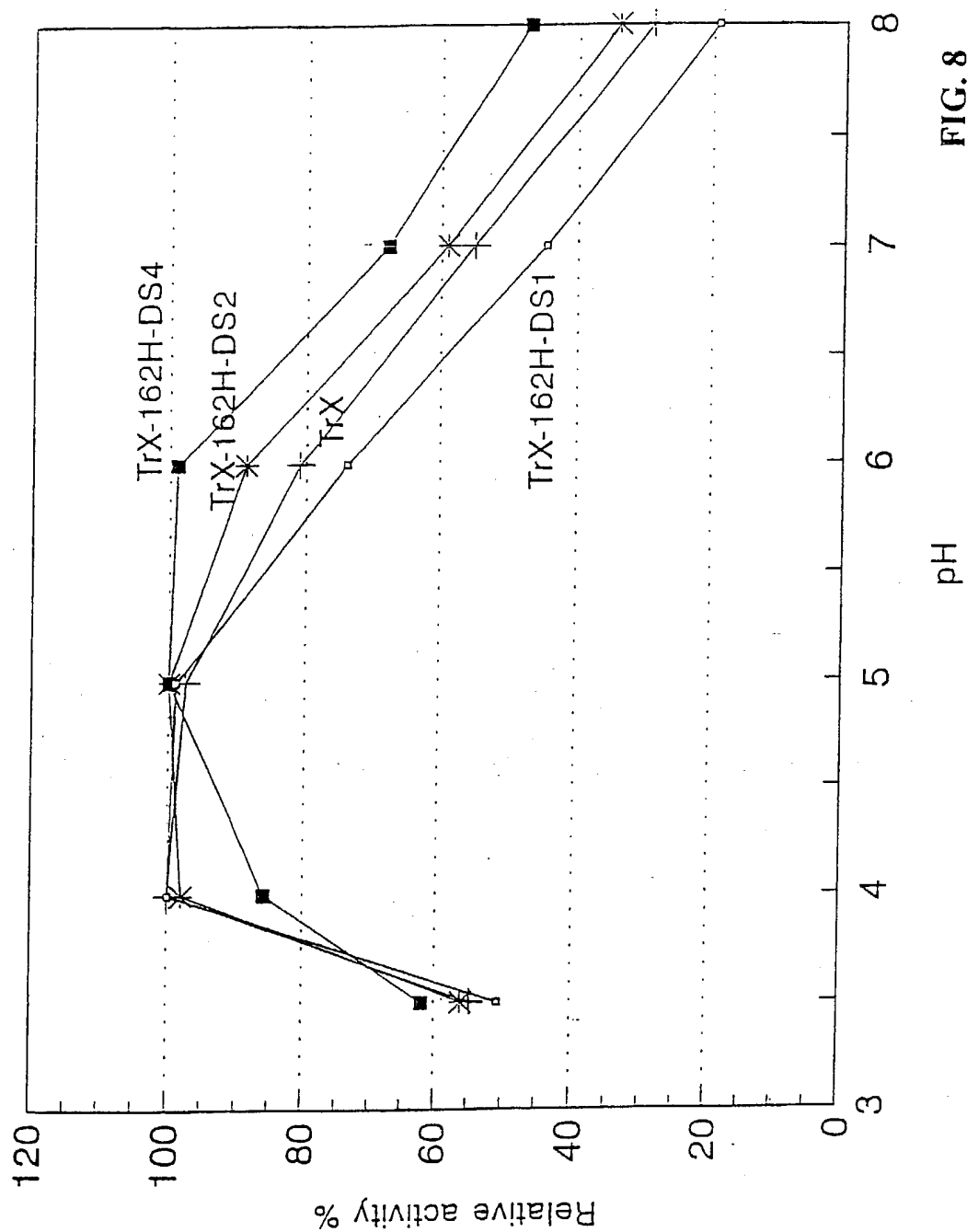
11/12

Effect of temperature on the hydrolysis of xylan by mutant Trx

pH 4.5, 30 min, 1.5 microgram of each enzyme



12/12



SUBSTITUTE SHEET (RULE 26)

1/28

SEQUENCE LISTING

<110> Wing Dr., Sung L.
Tolan Dr., Jeffrey S.

<120> Xylanases with Improved Performance in Feed Pelletting Applications

<130> 0888161US

<140>

<141>

<150> 60/108,504

<151> 1998-11-16

<160> 61

<170> PatentIn Ver. 2.1

<210> 1

<211> 184

<212> PRT

<213> *Aspergillus niger*

<400> 1

Ser Ala Gly Ile Asn Tyr Val Gln Asn Tyr Asn Gly Asn Leu Gly Asp
1 5 10 15

Phe Thr Tyr Asp Glu Ser Ala Gly Thr Phe Ser Met Tyr Trp Glu Asp
20 25 30

Gly Val Ser Ser Asp Phe Val Val Gly Leu Gly Trp Thr Thr Gly Ser
35 40 45

Ser Asn Ala Ile Thr Tyr Ser Ala Glu Tyr Ser Ala Ser Gly Ser Ser
50 55 60

Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gly Ala Glu Tyr
65 70 75 80

Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala Thr
85 90 95

Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys Thr
100 105 110

Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe Thr
115 120 125

Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val Thr

2/28

130 135 140
 Val Ala Asn His Phe Asn Phe Trp Ala Gln His Gly Phe Gly Asn Ser
 145 150 155 160
 Asp Phe Asn Tyr Gln Val Met Ala Val Glu Ala Trp Ser Gly Ala Gly
 165 170 175
 Ser Ala Ser Val Thr Ile Ser Ser
 180

 <210> 2
 <211> 185
 <212> PRT
 <213> Aspergillus tubingensis

 <400> 2
 Ser Ala Gly Ile Asn Tyr Val Gln Asn Tyr Asn Gln Asn Leu Gly Asp
 1 5 10 15
 Phe Thr Tyr Asp Glu Ser Ala Gly Thr Phe Ser Met Tyr Trp Glu Asp
 20 25 30
 Gly Val Ser Ser Asp Phe Val Val Gly Leu Gly Gly Trp Thr Thr Gly
 35 40 45
 Ser Ser Asn Ala Ile Thr Tyr Ser Ala Glu Tyr Ser Ala Ser Gly Ser
 50 55 60
 Ala Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gln Ala Glu
 65 70 75 80
 Tyr Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala
 85 90 95
 Thr Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys
 100 105 110
 Thr Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe
 115 120 125
 Thr Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val
 130 135 140
 Thr Val Ala Asn His Phe Asn Phe Trp Ala His His Gly Phe His Asn
 145 150 155 160
 Ser Asp Phe Asn Tyr Gln Val Val Ala Val Glu Ala Trp Ser Gly Ala
 165 170 175
 Gly Ser Ala Ala Val Thr Ile Ser Ser

SUBSTITUTE SHEET (RULE 26)

3/28

180

185

<210> 3

<211> 185

<212> PRT

<213> Bacillus circulans

<400> 3

Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
 1 5 10 15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
 20 25 30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
 35 40 45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
 50 55 60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
 65 70 75 80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
 85 90 95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Arg
 100 105 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
 115 120 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
 130 135 140

Thr Phe Thr Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
 145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
 165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
 180 185

<210> 4

<211> 201

<212> PRT

<213> Bacillus pumilus

<400> 4

4/28

Arg Thr Ile Thr Asn Asn Glu Met Gly Asn His Ser Gly Tyr Asp Tyr
 1 5 10 15
 Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ser Met Thr Leu Asn Asn Gly
 20 25 30
 Gly Ala Phe Ser Ala Gly Trp Asn Asn Ile Gly Asn Ala Leu Phe Arg
 35 40 45
 Lys Gly Lys Lys Phe Asp Ser Thr Arg Thr His His Gln Leu Gly Asn
 50 55 60
 Ile Ser Ile Asn Tyr Asn Ala Ser Phe Asn Pro Ser Gly Asn Ser Tyr
 65 70 75 80
 Leu Cys Val Tyr Gly Trp Thr Gln Ser Pro Leu Ala Glu Tyr Tyr Ile
 85 90 95
 Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Ala Tyr Lys Gly Ser
 100 105 110
 Phe Tyr Ala Asp Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Thr Arg Val
 115 120 125
 Asn Gln Pro Ser Ile Ile Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser
 130 135 140
 Val Arg Gln Thr Lys Arg Thr Ser Gly Thr Val Ser Val Ser Ala His
 145 150 155 160
 Phe Arg Lys Trp Glu Ser Leu Gly Met Pro Met Gly Lys Met Tyr Glu
 165 170 175
 Thr Ala Phe Thr Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val
 180 185 190
 Met Thr Asn Gln Leu Phe Ile Gly Asn
 195 200

<210> 5
 <211> 185
 <212> PRT
 <213> Bacillus subtilis

<400> 5
 Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
 1 5 10 15
 Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
 20 25 30

SUBSTITUTE SHEET (RULE 26)

5/28

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
35 40 45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
50 55 60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65 70 75 80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
85 90 95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
100 105 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
115 120 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
130 135 140

Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

<210> 6

<211> 211

<212> PRT

<213> Clostridium acetobutylicum

<400> 6

Ser Ala Phe Asn Thr Gln Ala Ala Pro Lys Thr Ile Thr Ser Asn Glu
1 5 10 15

Ile Gly Val Asn Gly Gly Tyr Asp Tyr Glu Leu Trp Lys Asp Tyr Gly
20 25 30

Asn Thr Ser Met Thr Leu Lys Asn Gly Gly Ala Phe Ser Cys Gln Trp
35 40 45

Ser Asn Ile Gly Asn Ala Leu Phe Arg Lys Gly Lys Lys Phe Asn Asp
50 55 60

Thr Gln Thr Tyr Lys Gln Leu Gly Asn Ile Ser Val Asn Tyr Asn Cys
65 70 75 80

SUBSTITUTE SHEET (RULE 26)

6/28

Asn Tyr Gln Pro Tyr Gly Asn Ser Tyr Leu Cys Val Tyr Gly Trp Thr
85 90 95

Ser Ser Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly Ser Trp
100 105 110

Arg Pro Pro Gly Gly Thr Ser Lys Gly Thr Ile Thr Val Asp Gly Gly
115 120 125

Ile Tyr Asp Ile Tyr Glu Thr Thr Arg Ile Asn Gln Pro Ser Ile Gln
130 135 140

Gly Asn Thr Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Thr Lys Arg
145 150 155 160

Thr Ser Gly Thr Ile Ser Val Ser Lys His Phe Ala Ala Trp Glu Ser
165 170 175

Lys Gly Met Pro Leu Gly Lys Met His Glu Thr Ala Phe Asn Ile Glu
180 185 190

Gly Tyr Gln Ser Ser Gly Lys Ala Asp Val Asn Ser Met Ser Ile Asn
195 200 205

Ile Gly Lys
210

<210> 7

<211> 206

<212> PRT

<213> Clostridium stercorarium

<400> 7

Gly Arg Ile Ile Tyr Asp Asn Glu Thr Gly Thr His Gly Gly Tyr Asp
1 5 10 15

Tyr Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ile Met Glu Leu Asn Asp
20 25 30

Gly Gly Thr Phe Ser Cys Gln Trp Ser Asn Ile Gly Asn Ala Leu Phe
35 40 45

Arg Lys Gly Arg Lys Phe Asn Ser Asp Lys Thr Tyr Gln Glu Leu Gly
50 55 60

Asp Ile Val Val Glu Tyr Gly Cys Asp Tyr Asn Pro Asn Gly Asn Ser
65 70 75 80

Tyr Leu Cys Val Tyr Gly Trp Thr Arg Asn Phe Leu Val Glu Tyr Tyr
85 90 95

SUBSTITUTE SHEET (RULE 26)

7/28

Ile Val Glu Ser Trp Gly Ser Trp Arg Pro Pro Gly Ala Thr Pro Lys
 100 105 110

Gly Thr Ile Thr Gln Trp Met Ala Gly Thr Tyr Glu Ile Tyr Glu Thr
 115 120 125

Thr Arg Val Asn Gln Pro Ser Ile Asp Gly Thr Ala Thr Phe Gln Gln
 130 135 140

Tyr Trp Ser Val Arg Thr Ser Lys Arg Thr Ser Gly Thr Ile Ser Val
 145 150 155 160

Thr Glu His Phe Lys Gln Trp Glu Arg Met Gly Met Arg Met Gly Lys
 165 170 175

Met Tyr Glu Val Ala Leu Thr Val Glu Gly Tyr Gln Ser Ser Gly Tyr
 180 185 190

Ala Asn Val Tyr Lys Asn Glu Ile Arg Ile Gly Ala Asn Pro
 195 200 205

<210> 8

<211> 211

<212> PRT

<213> Ruminococcus flavefaciens

<400> 8

Ser Ala Ala Asp Gln Gln Thr Arg Gly Asn Val Gly Gly Tyr Asp Tyr
 1 5 10 15

Glu Met Trp Asn Gln Asn Gly Gln Gly Gln Ala Ser Met Asn Pro Gly
 20 25 30

Ala Gly Ser Phe Thr Cys Ser Trp Ser Asn Ile Glu Asn Phe Leu Ala
 35 40 45

Arg Met Gly Lys Asn Tyr Asp Ser Gln Lys Lys Asn Tyr Lys Ala Phe
 50 55 60

Gly Asn Ile Val Leu Thr Tyr Asp Val Glu Tyr Thr Pro Arg Gly Asn
 65 70 75 80

Ser Tyr Met Cys Val Tyr Gly Trp Thr Arg Asn Pro Leu Met Glu Tyr
 85 90 95

Tyr Ile Val Glu Gly Trp Gly Asp Trp Arg Pro Pro Gly Asn Asp Gly
 100 105 110

Glu Val Lys Gly Thr Val Ser Ala Asn Gly Asn Thr Tyr Asp Ile Arg
 115 120 125

8/28

Lys Thr Met Arg Tyr Asn Gln Pro Ser Leu Asp Gly Thr Ala Thr Phe
 130 135 140
 Pro Gln Tyr Trp Ser Val Arg Gln Thr Ser Gly Ser Ala Asn Asn Gln
 145 150 155 160
 Thr Asn Tyr Met Lys Gly Thr Ile Asp Val Ser Lys His Phe Asp Ala
 165 170 175
 Trp Ser Ala Ala Gly Leu Asp Met Ser Gly Thr Leu Tyr Glu Val Ser
 180 185 190
 Leu Asn Ile Glu Gly Tyr Arg Ser Asn Gly Ser Ala Asn Val Lys Ser
 195 200 205
 Val Ser Val
 210

<210> 9
 <211> 197
 <212> PRT
 <213> Schizophyllum commune

<400> 9
 Ser Gly Thr Pro Ser Ser Thr Gly Thr Asp Gly Gly Tyr Tyr Tyr Ser
 1 5 10 15
 Trp Trp Thr Asp Gly Ala Gly Asp Ala Thr Tyr Gln Asn Asn Gly Gly
 20 25 30
 Gly Ser Tyr Thr Leu Thr Trp Ser Gly Asn Asn Gly Asn Leu Val Gly
 35 40 45
 Gly Lys Gly Trp Asn Pro Gly Ala Ala Ser Arg Ser Ile Ser Tyr Ser
 50 55 60
 Gly Thr Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp
 65 70 75 80
 Thr Arg Ser Ser Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Ser
 85 90 95
 Tyr Asp Pro Ser Ser Ala Ala Ser His Lys Gly Ser Val Thr Cys Asn
 100 105 110
 Gly Ala Thr Tyr Asp Ile Leu Ser Thr Trp Arg Tyr Asn Ala Pro Ser
 115 120 125
 Ile Asp Gly Thr Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro
 130 135 140

SUBSTITUTE SHEET (RULE 26)

9/28

Lys Lys Ala Pro Gly Gly Ser Ile Ser Gly Thr Val Asp Val Gln Cys
 145 150 155 160
 His Phe Asp Ala Trp Lys Gly Leu Gly Met Asn Leu Gly Ser Glu His
 165 170 175
 Asn Tyr Gln Ile Val Ala Thr Glu Gly Tyr Gln Ser Ser Gly Thr Ala
 180 185 190
 Thr Ile Thr Val Thr
 195
 <210> 10
 <211> 191
 <212> PRT
 <213> Streptomyces lividans
 <400> 10
 Asp Thr Val Val Thr Thr Asn Gln Glu Gly Thr Asn Asn Gly Tyr Tyr
 1 5 10 15
 Tyr Ser Phe Trp Thr Asp Ser Gln Gly Thr Val Ser Met Asn Met Gly
 20 25 30
 Ser Gly Gly Gln Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val
 35 40 45
 Ala Gly Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Gln Tyr Ser
 50 55 60
 Gly Ser Phe Asn Pro Ser Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp
 65 70 75 80
 Thr Ser Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Thr
 85 90 95
 Tyr Arg Pro Thr Gly Glu Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly
 100 105 110
 Thr Tyr Asp Ile Tyr Lys Thr Thr Arg Val Asn Lys Pro Ser Val Glu
 115 120 125
 Gly Thr Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg
 130 135 140
 Thr Gly Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
 145 150 155 160
 Ala Gly Met Pro Leu Gly Asn Phe Ser Tyr Tyr Met Ile Asn Ala Thr
 165 170 175

10/28

Glu Gly Tyr Gln Ser Ser Gly Thr Ser Ser Ile Asn Val Gly Gly
 180 185 190

<210> 11
 <211> 191
 <212> PRT
 <213> Streptomyces lividans

<400> 11
 Ala Thr Thr Ile Thr Thr Asn Gln Thr Gly Thr Asp Gly Met Tyr Tyr
 1 5 10 15

Ser Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly
 20 25 30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
 35 40 45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
 50 55 60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
 65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
 85 90 95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
 100 105 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Thr
 115 120 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
 130 135 140

Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
 145 150 155 160

Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Asn Ala Thr
 165 170 175

Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
 180 185 190

<210> 12
 <211> 189
 <212> PRT
 <213> Streptomyces sp.

SUBSTITUTE SHEET (RULE 26)

11/28

<400> 12

Ala Thr Thr Ile Thr Asn Glu Thr Gly Tyr Asp Gly Met Tyr Tyr Ser
 1 5 10 15

Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly Gly
 20 25 30

Gly Ser Tyr Ser Thr Arg Trp Thr Asn Cys Gly Asn Phe Val Ala Gly
 35 40 45

Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Arg Tyr Thr Gly Trp
 50 55 60

Phe Asn Pro Ser Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
 65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
 85 90 95

Pro Thr Gly Glu Thr Arg Gly Thr Val His Ser Asp Gly Gly Thr Tyr
 100 105 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Ala Pro
 115 120 125

Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
 130 135 140

Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly
 145 150 155 160

Met Asn Met Gly Asn Phe Arg Tyr Tyr Met Ile Asn Ala Thr Glu Gly
 165 170 175

Tyr Gln Ser Ser Gly Ser Ser Thr Ile Thr Val Ser Gly
 180 185

<210> 13

<211> 189

<212> PRT

<213> Thermomonospora fusca

<400> 13

Ala Val Thr Ser Asn Glu Thr Gly Tyr His Asp Gly Tyr Phe Tyr Ser
 1 5 10 15

Phe Trp Thr Asp Ala Pro Gly Thr Val Ser Met Glu Leu Gly Pro Gly
 20 25 30

Gly Asn Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly
 35 40 45

12/28

Lys Gly Trp Ala Thr Gly Gly Arg Arg Thr Val Thr Tyr Ser Ala Ser
50 55 60

Phe Asn Pro Ser Gly Asn Ala Tyr Leu Thr Leu Tyr Gly Trp Thr Arg
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Met Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr
115 120 125

Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr Ser
130 135 140

Gly Thr Ile Thr Ala Gly Asn His Phe Asp Ala Trp Ala Arg His Gly
145 150 155 160

Met His Leu Gly Thr His Asp Tyr Met Ile Met Ala Thr Glu Gly Tyr
165 170 175

Gln Ser Ser Gly Ser Ser Asn Val Thr Leu Gly Thr Ser
180 185

<210> 14

<211> 190

<212> PRT

<213> Trichoderma harzianum

<400> 14

Gln Thr Ile Gly Pro Gly Thr Gly Tyr Ser Asn Gly Tyr Tyr Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Ala Gly Val Thr Tyr Thr Asn Gly Gly Gly
20 25 30

Gly Ser Phe Thr Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Ile Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

SUBSTITUTE SHEET (RULE 26)

13/28

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
 100 105 110
 Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
 115 120 125
 Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
 130 135 140
 Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
 145 150 155 160
 Ser His Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
 165 170 175
 Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
 180 185 190

<210> 15

<211> 178

<212> PRT

<213> Trichoderma reesei

<400> 15

Ala Ser Ile Asn Tyr Asp Gln Asn Tyr Gln Thr Gly Gly Gln Val Ser
 1 5 10 15
 Tyr Ser Pro Ser Asn Thr Gly Phe Ser Val Asn Trp Asn Thr Gln Asp
 20 25 30
 Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
 35 40 45
 Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
 50 55 60
 Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
 65 70 75 80
 Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
 85 90 95
 Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
 100 105 110
 Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
 115 120 125
 Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
 130 135 140

14/28

Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Met Asn Tyr Gln Val
 145 150 155 160

Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
 165 170 175

Ser Asn

<210> 16

<211> 190

<212> PRT

<213> Trichoderma reesei

<400> 16

Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
 1 5 10 15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
 20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
 35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
 50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
 65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
 85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
 100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
 115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
 130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
 145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
 165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
 180 185 190

SUBSTITUTE SHEET (RULE 26)

15/28

<210> 17
 <211> 190
 <212> PRT
 <213> Trichoderma viride

<400> 17
 Gln Thr Ile Gln Pro Gly Thr Gly Phe Asn Asn Gly Tyr Phe Tyr Ser
 1 5 10 15
 Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
 20 25 30
 Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
 35 40 45
 Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
 50 55 60
 Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
 65 70 75 80
 Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
 85 90 95
 Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
 100 105 110
 Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
 115 120 125
 Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Thr His
 130 135 140
 Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
 145 150 155 160
 Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
 165 170 175
 Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
 180 185 190

<210> 18
 <211> 596
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: TrX synthetic
 sequence

16/28

<400> 18

ctagctaagg aggctgcaga tgcaaacaat acaaccagga accgggttaca acaacggtta 60
 cttttacagc tattggaacg atggccatgg tgggtgttacc tatacaaacg ggcccggagg 120
 ccaatttagc gtcaattggg ctaactccgg aaacttcgta ggtggaaaag gttggcaacc 180
 cgggaccaaa aataagggtga tcaacttctc tggatcttat aatccgaatg ggaattcata 240
 cttaagcgtc tatggctggg ctgaaaaccc actgattgaa tattacattg tcgaaaattt 300
 cggtagcctac aatccgagta ccggcgccac aaaattaggc gaagtcacta gtgatggatc 360
 cgtatatgat atctaccgta cccaacgcgt taatcagcca tcgatcattg gaaccgccac 420
 cttttatcag tactggagtg ttagacgtaa tcatcggagc tccggttcgg ttaatactgc 480
 gaatcacttt aatgcatggg cacagcaagg gttaacccta ggtacaatgg attatcaaat 540
 cgtagcggtg gaaggctact tctcgagtgg ttccgctagt attacagtga gctaaa 596

<210> 19

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Trx-110C
 Synthetic Sequence

<400> 19

atatacggat ccatacacaag tgacttcgcc taattttgtg 40

<210> 20

<211> 68

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Tx-110C-2

<400> 20

gcgccacaaa attaggcgaa gtcacttgtg atggatccgt atatgatatc taccgtaccc 60
 aacgcggtt 68

<210> 21

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Tx-103b

<400> 21

aatcagccat cgatcattgg aaccgccacc ttttatcagt ac 42

<210> 22

SUBSTITUTE SHEET (RULE 26)

17/28

<211> 54
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-109
Synthetic sequence

<400> 22
ggaggcggtt ccaatgatcg atggctgatt aacgcgttgg gtacggtaga tacc 54

<210> 23
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108b

<400> 23
cgaaccggag ctccgatgat tacgtctaac actccagtao tgataaaa 48

<210> 24
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-154C
Synthetic sequence

<400> 24
ctagggttaa cccttgatgat gccagggcat taaagtggca tgcagtatta ac 52

<210> 25
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-154C-2

<400> 25
tggagtgtta gacgtaatca tcggagctcc gggtcgggta atactgcatt ccactttaat 60
gcctgggcac agcaagggtt aacc 84

<210> 26
<211> 34

18/28

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-162H-3

<400> 26
ccacttcaat gcatgggcac agcacgggtt aacc

34

<210> 27
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-4

<400> 27
ctagggttaa cccgtgctgt gccatgcat tgaagtggca tg

42

<210> 28
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-101

<400> 28
tcgacaattt cggtagctac aatccgagta cggcgccac aaaattagga gaagtcac

58

<210> 29
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-102

<400> 29
tagtgatgga tccgtatatg atatctaccg tacccaacgc gttaatcagc ca

52

<210> 30
<211> 60
<212> DNA
<213> Artificial Sequence

<220>

SUBSTITUTE SHEET (RULE 26)

19/28

<223> Description of Artificial Sequence:TrX-103

<400> 30

tcgatcattg gaaccgccac cttttatcag tactggagtg ttagacgtaa tcatcggagc 60

<210> 31

<211> 69

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XyTv-104

<400> 31

tccgggttcgg ttaatactgc gaatcacttt aatgcatggg cacagcaagg gttaacccta 60
ggtacaatg 69

<210> 32

<211> 67

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XyTv-105

<400> 32

gattatcaaa tcgtagcggg ggaaggctac ttctcgagtg gtcccgctag tattacagtg 60
agctaaa 67

<210> 33

<211> 53

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XyTv-106
synthetic sequence

<400> 33

gatcttttagc tcaactgtaat actagcggaa ccactcgaga agtagccttc cac 53

<210> 34

<211> 66

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XyTv-107

20/28

<400> 34
cgctacgatt tgataatcca ttgtacctag ggttaaccct tgctgtgcc atgcattaaa 60
gtgatt 66

<210> 35
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-108

<400> 35
cgcagtatta accgaaccgg agctccgatg attacgtcta aactccagt actgataaaa 60

<210> 36
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-110

<400> 36
atatacggat ccatacctag tgacttcgcc taattttgtg gcgccggtac tcggattgta 60
ggtaccgaaa ttg 73

<210> 37
<211> 76
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-1

<400> 37
ctagctaagg aggctgcaga tgcaaacaaat acaaccagga accggttaca acaacggtta 60
cttttacagc tattgg 76

<210> 38
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-2

<400> 38

SUBSTITUTE SHEET (RULE 26)

21/28

aacgatggcc atggtggtgt tacctataca aacgggcccg gaggcccaatt tagcgtcaat 60
tggctctaact ccggaaac 78

<210> 39

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:TrX-3

<400> 39

ttcgtaggtg gaaaaggttg gcaacccggg accaaaaata aggtgatcaa cttctctgga 60
tcttataatc cgaatggg 78

<210> 40

<211> 74

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XyTv-4

<400> 40

aattcatact taagcgtcta tggctggtct agaaacccac cgattgaata ttacattgtc 60
gaaaatttcg gtac 74

<210> 41

<211> 85

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XyTv-5

<400> 41

gcaaattttc gacaatgtaa tattcaatca gtgggtttct agaccagcca tagacgetta 60
agtatgaatt cccattcgga ttata 85

<210> 42

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Trx-6Synthetic
sequence

22/28

<400> 42
agatccagag aagttgatca ccttattttt ggtcccgggt tgccaacctt ttccacctac 60
gaagtttccg gagttaga 78

<210> 43
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-7
Synthetic sequence

<400> 43
ccaattgacg ctaaattggc ctccggggccc gtttgtatag gtaacaccac catggccatc 60
gttccaatag ctgtaaaagt aacc 84

<210> 44
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-8 synthetic
sequence

<400> 44
gttggtgtaa ccggttcctg gttgtattgt ttgcacctgc agcctcctta g 51

<210> 45
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108C
synthetic sequence

<400> 45
atatacggat ccatacctag tgcattcgcc taattttgtg 40

<210> 46
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108C-2

SUBSTITUTE SHEET (RULE 26)

23/28

<400> 46

gcgccacaaa attagggcgaa tgcactagtg atggatccgt atatgatatc taccgtaccc 60
aacgcggt 68

<210> 47

<211> 52

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Tx-158C-162H
synthetic sequence

<400> 47

ctaggggttaa cccgtgtgat gccagcaat taaagtgatt tgcagtatta ac 52

<210> 48

<211> 84

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Tx-158C-162H-2

<400> 48

tggagtgtta gacgtaatca tcggagctcc ggttcggtta atactgcaaa tcactttaat 60
tgctgggcac agcacgggtt aacc 84

<210> 49

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Tx-108C-110C
synthetic sequence

<400> 49

atatacggat ccatacacaag tgcattcgcc taattttgtg 40

<210> 50

<211> 68

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Tx-108C-110C-2
synthetic sequence

24/28

<400> 50
 gcgccacaaa attagggcgaa tgcacttggtg atggatccgt atatgatatc taccgtaccc 60
 aacgcgtt 68

<210> 51
 <211> 52
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial
 Sequence:Tx-154C-158C-152H synthetic sequeunce

<400> 51
 ctagggttaa cccgtgtgat gccagcaat taaagtggca tgcagtatta ac 52

<210> 52
 <211> 84
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial
 Sequence:Tx-154C-158C-162H-2

<400> 52
 tggagtgtta gacgtaatca tcggagctcc ggttcgggtta atactgcatg ccactttaat 60
 tgctgggcac agcacgggtt aacc 84

<210> 53
 <211> 190
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:TrX amino acid
 sequence

<400> 53
 Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
 1 5 10 15
 Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
 20 25 30
 Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
 35 40 45
 Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly

SUBSTITUTE SHEET (RULE 26)

25/28

50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

```
<210> 54
<211> 198
<212> DNA
<213> Artificial Sequence
```

<220>
<223> Description of Artificial Sequence:TrX-DS1
cassette

```

<400> 54
gcgccacaaa attaggcgaa gtcacttgat atggatccgt atatgatatc taccgtagcc 60
aacgcgttaa tcagccatcg atcattggaa cgcgcacott ttatcagtag tggagtgtta 120
gacgtaatca tcggagctcc ggttcgggta atactgcatg ccactttaat gcctgggcac 180
agcaagggtt aaccctag
198

```

```
<210> 55
<211> 67
<212> PRT
<213> Artificial Sequence
```

<220>
<223> Description of Artificial Sequence:TrX-DS1
cassette aa

26/28

<400> 55

Gly Ala Thr Lys Leu Gly Glu Val Thr Cys Asp Gly Ser Val Tyr Asp
 1 5 10 15

Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile Ile Gly Thr Ala
 20 25 30

Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser Ser Gly
 35 40 45

Ser Val Asn Thr Ala Cys His Phe Asn Ala Trp Ala Gln Gln Gly Leu
 50 55 60

Thr Leu Gly
 65

<210> 56

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:TrX-162H-DS1
 cassette aa

<400> 56

Ala Cys His Phe Asn Ala Trp Ala Gln His Gly Leu Thr Leu Gly
 1 5 10 15

<210> 57

<211> 198

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:TrX-162H-DS2
 cassette

<400> 57

gcgccacaaa attaggcgaa tgcactagt atggatccgt atatgatatc taccgtaccc 60
 aacgcgttaa tcagccatcg atcattggaa ccgccacctt ttatcagtac tggagtgtta 120
 gacgtaatca tcggagctcc gggtcgggta atactgcaaa tcactttaat tgctggggcac 180
 agcacggggtt aaccctag 198

<210> 58

<211> 67

<212> PRT

<213> Artificial Sequence

SUBSTITUTE SHEET (RULE 26)

27/28

<220>

<223> Description of Artificial Sequence:TrX-162H-DS2
cassette aa

<400> 58

Gly Ala Thr Lys Leu Gly Glu Cys Thr Ser Asp Ser Ser Val Tyr Asp
1 5 10 15

Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile Ile Gly Thr Ala
20 25 30

Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser Ser Gly
35 40 45

Ser Val Asn Thr Ala Asn His Phe Asn Cys Trp Ala Gln His Gly Leu
50 55 60

Thr Leu Gly
65

<210> 59

<211> 198

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:TrX-162H-DS4
cassette

<400> 59

gcgccacaaa attaggcgaa tgcacttggtg atggatccgt atatgatatc taccgtaccc 60
aacgcgttaa tcagccatcg atcattggaa ccgccacctt ttatcagtac tggagtgtta 120
gacgtaatca tcggagctcc ggttcgggta atactgcatg ccactttaat tgctggggcac 180
agcacgggtt aaccctag 198

<210> 60

<211> 67

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:TrX-162H-DS4
cassete aa

<400> 60

Gly Ala Thr Lys Leu Gly Glu Cys Thr Cys Asp Gly Ser Val Tyr Asp
1 5 10 15

Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile Ile Gly Thr Ala
20 25 30

28/28

Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser Ser Gly
35 40 45

Ser Val Asn Thr Ala Cys His Phe Asn Cys Trp Ala Gln His Gly Leu
50 55 60

Thr Leu Gly
65

<210> 61

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: TrX-162H-DS1
cassette

<400> 61

catgccactt caatgcatgg gcacagcacg gggttaaccct ag

42

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/01093

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/56 C12N9/24 A23K1/165

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 828 002 A (NATIONAL RESEARCH COUNCIL OF CANADA) 11 March 1998 (1998-03-11) cited in the application	1,2, 9-11, 23-28
Y	page 6, line 10 - line 13; example 12	15-22
Y	WAKARCHUK W W ET AL: "THERMOSTABILIZATION OF THE BACILLUS CIRCULANS XYLANASE BY THE INTRODUCTION OF DISULFIDE BONDS" PROTEIN ENGINEERING, GB, OXFORD UNIVERSITY PRESS, SURREY, vol. 7, no. 11, 1 January 1994 (1994-01-01), pages 1379-1386, XP002072553 ISSN: 0269-2139 cited in the application the whole document	15-22

-/-

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

22 February 2000

Date of mailing of the international search report

09/03/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/01093

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29997 A (FINNFEEDS INT LTD ;MORGAN ANDREW JOHN (GB); BODIE ELIZABETH A (US)) 9 November 1995 (1995-11-09) page 4, line 19 -page 9, line 13; example 1 ---	1,2,9, 10,15, 16,25-28
X	WO 94 24270 A (NATIONAL RESEARCH COUNCIL OF CANADA; CAMPBELL RL ET AL.) 27 October 1994 (1994-10-27) cited in the application page 160 -page 163; claim 5 ---	1,2,9, 10,15, 16,23,24
X	MOREAU A ET AL: "INCREASE IN CATALYTIC ACTIVITY AND THERMOSTABILITY OF THE XYLANASE A OF STREPTOMYCES LIVIDANS 1326 BY SITE-SPECIFIC MUTAGENESIS" ENZYME AND MICROBIAL TECHNOLOGY,US,STONEHAM, MA, vol. 16, no. 5, 1 May 1994 (1994-05-01), pages 420-424, XP002072550 ISSN: 0141-0229 page 424, left-hand column, paragraph 2 ---	1,2,6,9, 10,23,24
X	GRUBER K ET AL: "Thermophilic xylanase from Thermomyces lanuginosus: high-resolution X-ray structure and modeling studies" BIOCHEMISTRY, vol. 37, no. 29, 29 September 1998 (1998-09-29), pages 13475-13485, XP002131131 EASTON, PA US page 13483 -----	1-3,6,10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 99/01093

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0828002 A	11-03-1998	US 5759840 A	02-06-1998
		CA 2210247 A	09-03-1998
		JP 10179155 A	07-07-1998
		NZ 328680 A	27-04-1998
		US 5866408 A	02-02-1999
WO 9529997 A	09-11-1995	US 5437992 A	01-08-1995
		AU 681031 B	14-08-1997
		AU 2394995 A	29-11-1995
		AU 684026 B	27-11-1997
		AU 2448595 A	29-11-1995
		BR 9507540 A	05-08-1997
		CA 2188874 A	09-11-1995
		CN 1147271 A	09-04-1997
		CZ 9603155 A	14-05-1997
		EP 0758378 A	19-02-1997
		EP 0758379 A	19-02-1997
		EP 0926234 A	30-06-1999
		FI 964283 A	24-10-1996
		FI 964319 A	25-10-1996
		HU 75662 A	28-05-1997
		JP 9512709 T	22-12-1997
		NO 964549 A	23-12-1996
		NZ 285406 A	22-09-1997
		PL 316997 A	03-03-1997
		WO 9529998 A	09-11-1995
		US 5683911 A	04-11-1997
		ZA 9503458 A	17-01-1996
WO 9424270 A	27-10-1994	US 5405769 A	11-04-1995



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/56, 9/24, A23K 1/165		A1	(11) International Publication Number: WO 00/29587
			(43) International Publication Date: 25 May 2000 (25.05.00)
(21) International Application Number: PCT/CA99/01093		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 16 November 1999 (16.11.99)			
(30) Priority Data: 60/108,504 16 November 1998 (16.11.98) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/108,504 (CON) Filed on 16 November 1998 (16.11.98)			
(71) Applicant (for all designated States except US): IOGEN CORPORATION [CA/CA]; 400 Hunt Club Road, Ottawa, Ontario K1V 1C1 (CA).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only): SUNG, Wing, L. [CA/CA]; 2148 Fillmore Crescent, Gloucester, Ontario K1J 6A4 (CA). TOLAN, Jeffrey, S. [CA/CA]; 114 Hawk Crescent, Ottawa, Ontario K1V 9G7 (CA).			
(74) Agents: SECHLEY, Konrad, A. et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).			

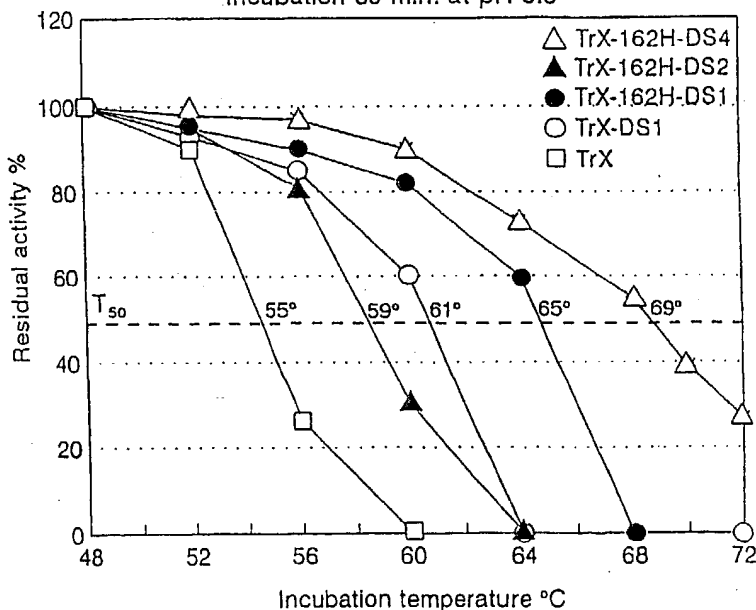
(54) Title: THERMOSTABLE XYLANASES

(57) Abstract

The present invention is directed to thermostable xylanase enzymes that are suitable for feed pelleting applications. The novel xylanase enzymes comprise at least 40 % of their optimal activity from a pH range from about pH 3.5 to about pH 6.0, and from about 40 to about 60 °C, and exhibit at least 30 % of their optimal activity after a pre-incubation step for 30 minutes at 70 °C in the presence of 40 % glycerol. Also disclosed are modified xylanase molecules comprising either a basic amino acid at position 162 (TrX numbering), or its equivalent position in other xylanase molecules, at least one disulfide bridge, or a combination thereof. The thermostable xylanase molecules of the present invention have a physiological temperature and pH optima and are useful as animal feeds additives since they can withstand the heat associated with feed sterilization and pellet formation, yet they exhibit optimal activity within an animal to aid in breakdown of ingested feed.

Thermostability of mutant TrX

Incubation 30 min. at pH 5.5



*(Referred to in PCT Gazette No. 38/2000, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		